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<p>(21) International Application Number: <b>PCT/IL99/00417</b> (22) International Filing Date: <b>29 July 1999 (29.07.99)</b>  (30) Priority Data: 125608 30 July 1998 (30.07.98) <b>IL</b>  (71) Applicants (for all designated States except US): <b>YEDA RESEARCH AND DEVELOPMENT COMPANY LTD AT THE WEIZMANN INSTITUTE OF SCIENCE [IL/IL]; P.O. Box 95, 76100 Rehovot (IL). BIO-TECHNOLOGY GENERAL CORP. [US/US]; 70 Wood Avenue South, Iselin, NJ 08830 (US).</b>  (72) Inventors; and (75) Inventors/Applicants (for US only): <b>EISENBACH, Lea [IL/IL]; 33 Hanasi Harishon Street, 76303 Rehovot (IL). CARMON, Lior [IL/IL]; 6 Kehilat Kiyov Street, 69989 Tel Aviv (IL). TIROSH, Boaz [IL/IL]; 9 Stern Street, 55602 Kiryat Ono (IL). BAR-HAIM, Erez [IL/IL]; 25/8 Hatsivony Street, 81573 Yavne (IL). PAZ, Adrian [IL/IL]; 25 Wolfson Street, 49541 Petach Tikva (IL). FRIDKIN, Matityahu [IL/IL]; 23 Miller Street, 76284 Rehovot (IL). FITZER-ATTAS, Cheryl [US/IL]; 9 Sapir Street, 76227 Rehovot (IL).</b></p>		<p>(74) Agents: <b>SCHUZ, David et al.; Bio-Technology General (Israel) Ltd., Patent Dept., Kiryat Weizmann, 76326 Rehovot (IL).</b>  (81) Designated States: <b>AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</b>  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: <b>TUMOR ASSOCIATED ANTIGEN PEPTIDES AND USE OF SAME AS ANTI-TUMOR VACCINES</b>  (57) Abstract  The present invention relates to tumor associated antigen (TAA) peptides and to the use of same, of polynucleotides encoding same and of cells presenting same as anti-tumor vaccines. More particularly, the present invention relates to tumor associated antigen peptides derived from Uroplakin Ia, Ib, II and III, Prostate specific antigen (PSA), Prostate acid phosphatase (PAP) and Prostate specific membrane antigen (PSMA), BA-46 (Lactadherin), Mucin (MUC-1), and Teratocarcinoma-derived growth factor (CRIPTO-1) and the use of same as anti-tumor vaccines to prevent or cure bladder, prostate, breast or other cancers, carcinomas in particular. Most particularly, the present invention relates to tumor associated antigen peptides which are presentable to the immune system by HLA-A2 molecules.</p>		

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## TUMOR ASSOCIATED ANTIGEN PEPTIDES AND USE OF SAME AS ANTI-TUMOR VACCINES

### FIELD AND BACKGROUND OF THE INVENTION

5       The present invention relates to tumor associated antigen (TAA) peptides and to the use of same, of polynucleotides encoding same and of cells presenting same as anti-tumor vaccines. More particularly, the present invention relates to tumor associated antigen peptides derived from Uroplakin Ia, Ib, II and III, Prostate specific antigen (PSA), Prostate acid phosphatase (PAP) and Prostate  
10 specific membrane antigen (PSMA), BA-46 (Lactadherin), Mucin (MUC-1), and Teratocarcinoma-derived growth factor (CRIPTO-1) and the use of same as anti-tumor vaccines to prevent or cure bladder, prostate, breast or other cancers, carcinomas in particular. Most particularly, the present invention relates to tumor associated antigen peptides which are presentable to the immune system by  
15 HLA-A2 molecules.

Local therapy such as surgical excision or ablation by radiation is a mainstay for the treatment of primary cancer and is curative for a percentage of patients. However, many malignancies will recur locally or at a distant site. Thus the prevention or cure of metastases remains a major focus in clinical oncology  
20 (1). Although early detection followed by surgery provides good prognosis for a number of major cancer types, a large fraction of patients would need adjuvant therapy. Part of these patients will, with time, succumb to metastasis (2-4). Alternative approaches based on gene therapy and immunotherapy have been the focus of attention in the last years. One such approach is specific active  
25 immunotherapy (SAI, 5). The objective of SAI is to stimulate a tumor specific cytotoxic T lymphocytes (CTL) immune response that is capable of eliminating residual metastatic disease and induce a state of immunity to protect the patients from recurrent disease. The underlying assumption of SAI is that tumor cells express tumor antigens which are sufficiently distinct in structure or context to  
30 induce an effective CTL response (6). Although the validity of these assumptions was questioned, a number of studies in the last decade have demonstrated the rational of SAI. In a landmark study, van Pel and Boon have shown that tumor associated antigens (TAAs) can be isolated and defined (7). Importantly, *ex-vivo* manipulations of "non-immunogenic" animal tumor cells can be used to elicit  
35 effective immune responses which will also recognize parental "non-immunogenic" tumor cells (8). Studies employing rodent tumor models with little intrinsic immunogenicity have shown that genetically modified tumor cells transduced to express MHC class I, cytokines such as IL-1, IL-2, IL-4, IL-6, IL-7, IL-12, IFN or GM-CSF or costimulatory molecules such as B7-1 or B7-2 were

capable of preventing or causing regression of tumors or metastases (reviewed in 9). Although gene modified tumor vaccine (GMTV) clinical trials, with improved retroviral vectors or other transfer methodologies are currently tested, it becomes clear that GMTV using autologous tumor cells might be limited by its complexity, high cost and ineffective gene transfer methodologies (10). One alternative approach would be vaccination with tumor associated antigens (TAAs) presented in an effective way to the patient's immune system, to induce antigen specific CTL (11).

Cytotoxic T lymphocytes (CTL), directed against peptides presented by MHC class I molecules, constitute powerful effectors of the immune system against tumors or infectious agents (12). These peptides are usually 8-10 amino acids long with 2-3 primary anchor residues that interact with the MHC class I molecules and 2-3 amino acid residues which bind to the T cell receptor (13). Several methods have been employed to identify CTL epitopes. If the amino acid sequence of a protein antigen is known, like in the case of viral proteins, oncogenes, suppressor genes or growth factor receptors, overlapping peptides of 8-10 amino acids in length can be synthesized and screened as CTL targets (14). CTL epitopes may also be identified subsequent to the search for MHC binding motifs in known proteins (15). If the tumor antigen is not known, isolation of the TAA peptides from total acid extract or from MHC class I molecules followed by HPLC fractionation steps and Edman sequencing (16) or mass spectrometry (17) provide a direct way of identifying CTL epitopes. Recently, a synthetic combinatorial library approach, in which defined amino acids in two MHC anchor positions are fixed and all other positions are subgrouped for CTL screening has led to the description of novel EL4 TAA peptide mimotopes (18).

The most fruitful method, so far, designed by T. Boon and his colleagues is the genetic approach in which cDNA expression libraries are pool transfected into COS7 cells with the appropriate HLA and screened by CTL lines. This approach led to the discovery of several human melanoma and mouse mastocytoma antigens recognized by specific CTL (19). The first report of a phase I clinical trial with the synthetic MAGE3 melanoma peptide, restricted by HLA-A1, showed regression of cutaneous, subcutaneous and lung metastases in 3/6 patients (20). Recently, two reports of clinical trials have shown that treatment of patients with a melanoma gp100 TAA peptide together with IL-2 resulted in significant tumor regression in 13/31 (42 %) patients and that vaccination with defined peptides or total peptide extracts on autologous dendritic cells (DC) resulted in complete or partial cures (21, 22). Regression of lung carcinoma established metastases or small established tumors was demonstrated in a murine model by peptide



vaccination (23, 24). These observations suggest that TAA peptide vaccines may constitute a reasonable therapeutic modality in advanced cancer. In studies with murine tumors, CTL are induced *in vivo* by immunization with irradiated tumor cells, often gene modified by MHC class I; cytokine or costimulatory molecules like B7-1 or B7-2 genes (16, 18, 25). In melanomas, CTL lines were mostly induced from peripheral blood mononuclear cells (PBMC) of patients or from tumor infiltrated lymphocytes (TIL, 19, 26). Yet, most metastatic tumors are non-immunogenic tumors and it is extremely difficult to derive CTL lines or clones from TIL or patient's PBL. Moreover, *in vitro* propagated CTL clones do not always represent dominant anti-tumor specificities but rather sporadic clones surviving culture conditions. Lately, a number of studies have compared the CTL repertoire of viral or other defined peptides, restricted by HLA-A2.1 in human PBL from HLA-A2.1 expressing patients to CTL induced in HLA-A2.1 transgenic mice. Good concordance between human HLA-A2.1 and murine transgenic HLA-A2.1 CTL repertoire was found, confirming the potential of such transgenics in identification of human CTL epitopes (27). Although vaccination with defined peptides of HLA transgenic mice shows an overlapping repertoire to human CTL, vaccination of such mice with multi-epitope proteins shows that murine H-2 restricted responses are dominant and obliterate, as a rule, cytolytic responses with direct recognition of human HLA (28). Thus, by combining classical HLA class I transgenesis with selective destruction of murine H-2, it is possible to derive useful mouse strains for the study of HLA class I restricted responses. While reducing the present invention to practice, we utilized such mice for preparation of anti-tumor CTL as a tool for TAA purification and as a model system to assess the immunogenicity of peptides.

Murine H-2 knockout mice transgenic for a single human HLA seem to be a suitable model for induction of anti-tumor CTL. Classical  $\beta_2$  microglobulin knockout mice ( $\beta_2m^{-/-}$ ) do not express H-2K<sup>b</sup> or other non-classical class I molecules, yet they express low levels of H-2D<sup>b</sup> heavy chain in the absence of  $\beta_2m$ . To derive fully H-2 knockout mice, Prof. F. Lemonnier (Pasteur Institute, Paris), prepared H-2D<sup>b</sup><sup>-/-</sup> mice. These mice were crossed with  $\beta_2m^{-/-}$  mice and bred to derive homozygous  $\beta_2m^{-/-}$ , D<sup>b</sup><sup>-/-</sup> mice that do not express any H-2 class I. These mice are practically depleted of CD8<sup>+</sup> splenocytes, as well as other CD8<sup>+</sup> cells. To reconstitute in these mice expression of a stable HLA-A2.1, expression of  $\beta_2m$  is necessary. A construct containing a leader sequence, domains  $\alpha 1$  and  $\alpha 2$  of HLA-A2.1 and  $\alpha 3$ , transmembrane and cytoplasmic domains of H-2D<sup>b</sup> fused to human  $\beta_2m$  (HhD) was prepared. The exchange of the  $\alpha 3$  human domain by a murine domain in HhD is thought to improve the interaction of the class I

molecule with CD8 molecules of the murine CTL (29). This HhD construct was transfected into RMA and RMA-S cells and shown to bind HLA-A2.1 restricted peptides. The HhD construct was used to produce transgenic mice in C57BL/6 recipients and positive founder mice were bred to the  $\beta_2m^{-/-}$ ,  $Db^{-/-}$  mice (30).

5       The  $\beta_2m^{-/-}$ ,  $Db^{-/-}$ , HhD $^{-/+}$  heterozygous mice show reconstitution of CD8 $^{+}$  cells in the periphery relative to  $\beta_2m^{-/-}$   $Db^{-/-}$  mice. Moreover, preliminary data from Prof. Lemonnier's lab showed that CTL induced in HhD mice against influenza NP are directed to the same HLA-A2 dominant epitope as in the human repertoire. Homozygous HhD mice were derived and a colony was established in  
10       the Weizmann Institute of Science, Israel.

#### SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a peptide derived from a protein selected from the group consisting of Uroplakin (UP),  
15       Prostate specific antigen (PSA), Prostate specific membrane antigen (PSMA), Prostate acid phosphatase (PAP), Lactadherin (BA-46) and Mucin (MUC1), and Teratocarcinoma-derived growth factor (CRPTO-1), the peptide comprising 8 to 10 amino acid residues, of which a second residue from an amino terminal of the peptide and a carboxy terminal residue of the peptide are hydrophobic or  
20       hydrophilic natural or non natural amino acid residues, for example, SEQ ID NOS:1-64 and 65 to 77.

According to further features in preferred embodiments of the invention described below, the peptide is derived from Uroplakin, such as Uroplakin II, Uroplakin Ia, Uroplakin III and Uroplakin Ib, for example, SEQ ID NOS:1-19 or  
25       50-64.

According to still further features in the described preferred embodiments the peptide is derived from the Prostate specific antigen (PSA), for example, SEQ ID NOS:20-24.

According to still further features in the described preferred embodiments  
30       the peptide is derived from the Prostate specific membrane antigen (PSMA), for example, SEQ ID NOS:25-30.

According to still further features in the described preferred embodiments the peptide is derived from the Prostate acid phosphatase (PAP), for example, SEQ ID NOS:31-34.

35       According to still further features in the described preferred embodiments the peptide is derived from the Mucin, for example, SEQ ID NOS:42-49.

According to still further features in the described preferred embodiments the peptide is derived from a non tandem repeat array of the Mucin.

According to still further features in the described preferred embodiments the peptide is derived from a region selected from the group consisting of a signal peptide, a cytoplasmic domain and an extracellular domain of the Mucin.

According to still further features in the described preferred embodiments the peptide is derived from the Lactadherin (BA-46), for example, SEQ ID NOs:35-41.

According to still further features in the described preferred embodiments, the peptide is derived from the Teratocarcinoma-derived growth factor (CRIPTO-1), for example, SEQ ID NOs. 66 to 77.

According to still further features in the described preferred embodiments the Uroplakin (UP), Prostate specific antigen (PSA), Prostate specific membrane antigen (PSMA), Prostate acid phosphatase (PAP), Lactadherin (BA-46) and Mucin (MUC1) and Teratocarcinom-derived growth factor (CRIPTO-1) are each independently of mammalian origin.

According to still further features in the described preferred embodiments the mammal is selected from the group consisting of a humanoid and a rodent.

According to still further features in the described preferred embodiments the peptide includes at least one non-natural modification.

According to still further features in the described preferred embodiments the peptide includes at least one non-natural modification rendering peptides more immunogenic or more stable.

According to still further features in the described preferred embodiments the at least one modification is selected from the group consisting of peptoid modification, semipeptoid modification, cyclic peptide modification, N terminus modification, C terminus modification, peptide bond modification, backbone modification and residue modification.

According to another aspect of the present invention there is provided a pharmaceutical composition comprising, as an active ingredient, at least one peptide at set forth herein and a pharmaceutically acceptable carrier.

According to another aspect of the present invention there is provided a vaccine composition comprising, as an active ingredient, at least one peptide at set forth herein and a vaccination acceptable carrier.

According to still further features in the described preferred embodiments the carrier is selected from the group consisting of a proteinaceous carrier to which the at least one tumor associated antigen peptide is linked, an adjuvant, a protein or a recombinant protein and an antigen presenting cell.

According to still further features in the described preferred embodiments the composition, pharmaceutical or vaccine, is effective in prevention or cure of cancer or carcinoma metastases.

According to still further features in the described preferred embodiments  
5 the cancer is selected from the group consisting of breast, bladder, prostate, pancreas, ovary, thyroid, colon, stomach and head and neck cancer.

According to still further features in the described preferred embodiments the cancer is a carcinoma.

Further according to the present invention there is provided a method of  
10 prevention or cure of a cancer or of metastases thereof comprising the step of administering to a patient an effective amount of the pharmaceutical composition described herein.

Still further according to the present invention there is provided a method  
15 of prevention or cure of a cancer or of metastases thereof comprising the step of vaccinating a patient with an effective amount of the vaccine composition described herein.

According to yet another aspect of the present invention there is provided a polynucleotide encoding at least one peptide as set forth herein. One ordinarily skilled in the art would know how to reverse translate any of the peptides  
20 according to the present invention such as the peptides set forth in SEQ ID NOs:1-64 and 66 to 77, using, for example, the human preferred codon usage, to derive the sequences of the polynucleotides according to the present invention. Such polynucleotides are readily synthesizable using the well known solid phase technology for preparation of oligonucleotides such as oligodeoxynucleotides or  
25 analogs thereof.

According to still further features in the described preferred embodiments the polynucleotide forms a part of a longer polynucleotide designed to encode a fused protein product from which at least one peptide is cleavable by a protease.

According to a further aspect of the present invention there is provided a  
30 pharmaceutical composition comprising, as an active ingredient, at least one polynucleotide as set forth herein and a pharmaceutically acceptable carrier.

According to still a further aspect of the present invention there is provided a cellular vaccine composition comprising an antigen presenting cell presenting at least one peptide derived from a protein selected from the group consisting of  
35 Uroplakin (UP), Prostate specific antigen (PSA), Prostate specific membrane antigen (PSMA), Prostate acid phosphatase (PAP), Lactadherin (BA-46) Mucin (MUC1) and Teratocarcinoma-derived growth factor (CRIPTO-1), the at least one peptide comprising 9 or 10 amino acid residues, of which a second residue from

an amino terminal of the peptide and a carboxy terminal residue of the peptide are hydrophilic or aliphatic non-charged natural or synthetic amino acid residues.

According to still further features in the described preferred embodiments the antigen presenting cell is selected from the group consisting of a dendritic cell,  
5 a macrophage, a B cell and a fibroblast.

According to still further features in the described preferred embodiments the antigen presenting cell is caused to present the at least one tumor associated antigen peptide by a method selected from the group consisting of (a) genetically modifying the antigen presenting cell with at least one polynucleotide encoding  
10 the at least one tumor associated antigen peptide such that the said peptide or at least one longer polypeptide including said peptide will be expressed; (b) loading the antigen presenting cell with at least one polynucleotide encoding the at least one tumor associated antigen peptide; (c) loading the antigen presenting cell with the at least one tumor associated antigen peptide; and (d) loading the antigen  
15 presenting cell with at least one longer polypeptide including the at least one tumor associated antigen peptide.

According to a still further aspect of the present invention there is provided a pharmaceutical composition also comprising a helper peptide.

According to a still further aspect of the present invention there is provided  
20 a pharmaceutical composition, wherein the helper peptide has a T helper epitope.

According to a still further aspect of the present invention there is provided a vaccine composition also comprising a helper peptide.

According to a still further aspect of the present invention there is provided a vaccine composition, wherein the helper peptide has a T helper epitope.

25 According to a still further aspect of the present invention there is provided a use of the at least one peptide in the manufacture of a medicament.

According to a still further aspect of the present invention there is provided the at least one peptide for use as a medicament.

According to a still further aspect of the present invention there is provided  
30 a use of the at least one peptide in the manufacture of a medicament for the prevention or cure of a cancer or cancer metastases.

According to a still further aspect of the present invention there is provided the at least one peptide for use as a medicament for the prevention or cure of a cancer or cancer metastases.

35 According to a still further aspect of the present invention there is provided a peptide derived from a protein selected from the group consisting of Uroplakin (UP), Prostate specific antigen (PSA), Prostate specific membrane antigen (PSMA), Prostate acid phosphatase (PAP), Lactadherin (BA-46) and Mucin

(MUC1) and Teratocarcinoma-derived growth factor (CRIPTO-1), the peptide comprising 8-10 amino acid residues as selected so as to promote effective binding to a MHC class I type molecule such that a CTL response is elicitable.

The present invention successfully addresses the shortcomings of the presently known configurations by providing novel tumor associated antigen peptides effective in eliciting CTL response which may therefore be effective therapeutic agents to combat cancer.

#### BRIEF DESCRIPTION OF THE DRAWINGS

The invention herein described, by way of example only, with reference to the accompanying drawings, wherein:

FIG. 1 demonstrates stabilization of cell surface HhD on RMA-S cells by human Uroplakin II derived peptides. Uroplakin II derived synthetic peptides (HURO1-7) or a peptide from the melanoma associated TAA Tyrosinase (Tyr) which served as a control were loaded at various concentrations (3-300  $\mu$ M) on the RMA-HhD cells as described. Indirect fluorescence activated cell sorter (FACS) analyses were performed by incubating  $5 \times 10^5$  loaded cells with the anti-HLA-A2 monoclonal antibodies BB7.2 for 30 minutes at 4° C. After washing the cells in PBS-0.5 %, BSA-0.1 %, sodium azide, the second antibody, goat anti mouse-FITC was applied for 30 minutes at 4° C. Following another wash the fluorescence was recorded on a FACS scan (BD). Mean fluorescence at 300  $\mu$ M is shown.

FIG. 2 demonstrates lytic activity of human peripheral blood lymphocytes (PBL) against Uroplakin II loaded target cells. PBL from HLA-A2 positive patients were resensitized with tumor extracted peptide preparations from transitional cell carcinoma (TCC) specimens as described in Materials and Methods. Resensitized lymphocytes from 4 TCC patients treated with Bacillus Calmette Guerein (BCG), 2 non-treated TCC patients, and 1 prostate cancer patient (control), were admixed at different ratios with labeled T2 cells, loaded with TCC extracted peptides (TCC pool) normal bladder mucosa extracted peptides (bladder), synthetic Uroplakin II peptides (HURO1-7) or the melanoma peptide from Tyrosinase (Tyr). Labeled target cells, J82, a HLA-A2 expressing TCC line, SUP, a low class I expressor and K562, a NK sensitive line were also tested. Percent specific lysis, at effector: target of 100:1 is presented.

FIG. 3 demonstrates lytic activity of human peripheral blood lymphocytes against peptides extracted from individual TCC tumors. PBL were resensitized as in Figure 2. Peptides extracted from 9 individual tumors (TCC1-9), that were

pooled for resensitization, were loaded on T2 cells. Labeled loaded targets were tested for lysis by sensitized PBL as described in Figure 2.

FIG. 4 demonstrates lysis of human Uroplakin II and murine Uroplakin II peptide loaded target cells by CTL induced against human Uroplakin II derived peptides in HhD mice. Mice were immunized as described, by RMA-S-HhD-B7.1 cells, loaded with human Uroplakin II derived synthetic peptides 1-7 as described in Materials and Methods. Loading was performed with individual peptides and cells were pooled for immunization. Resensitized CTL (see Materials and Methods) were tested for lysis of RMA-S-HhD targets loaded with human or murine Uroplakin II peptides (HURO 1-7, MURO1, 3, 4, 6) with TCC or normal bladder extracted peptides (RMA-S TCC, RMA-S bladder), with the tyrosinase peptide (RMA-S Tyr) with peptides derived from breast tumors (RMA-S Br.) or with the cell lines J82 or SUP. E:T ratios of 100:1, 50:1, 25:1 and 12.5:1 were tested and the results are presented in lytic units LU30 was calculated by linear regression analysis of percentage lysis vs. the log (ln) of effector cell number and expressed as the number of LU per  $10^6$  effector cells. The coefficient was 0.85 - 0.95 for all groups.

FIG. 5 demonstrates lysis of human Uroplakin II and Ia and murine Uroplakin II derived peptide loaded target cells by CTL induced against TCC extracted peptides in HhD mice. HhD mice were immunized by RMA-S-HhD-B7.1 cells loaded with peptides extracted from 9 TCC samples and pooled. CTL were prepared as described and tested for lysis of labeled RMA-S-HhD target cells loaded with synthetic, tissue derived or control peptides and cell lines as in Figure 4 and synthetic peptides derived from Uroplakin Ia (HURO 11-18). The results are presented in lytic units as described in Figure 4.

FIG. 6 demonstrates stabilization of cell surface HhD on RMA-S cells by human Prostate specific antigen (PSA) and Prostate acid phosphatase (PAP) derived peptides. PSA and PAP derived peptides were loaded at various concentrations (1  $\mu$ M - 1 mM) on RMA-S-HhD cells. FACS analysis was performed as described in Figure 1, wherein Tyr served as a control. Mean fluorescence at 1 mM is presented.

FIG. 7 demonstrates stabilization of cell surface HhD on RMA-S cells by human Prostate specific membrane antigen (PSMA) derived peptides. PSMA derived peptides (1  $\mu$ M - 1 mM) were loaded and cells were monitored as described in Figure 6.

FIG. 8 demonstrates immunogenicity and antigenicity of PAP derived peptides in HhD mice. HhD mice were immunized as described, by RMA-S-HhD-B7.1 cells loaded individually with each PAP peptide and pooled

before vaccination. CTL were tested against RMA-HhD cells loaded with synthetic peptides (PAP 1-4), prostate carcinoma extracted peptides (RMA-S, CAP), hyperplastic (normal) prostate extracted peptides (RMA-S HP), breast carcinoma derived peptides (RMA-S Br, control) and tyrosinase (RMA-S Tyr, control). The prostate carcinoma cell line DU145 (DU) and its HhD transfectant (DU HhD) were also tested as targets. The results are presented in lytic units as in Figure 4.

FIG. 9 demonstrates immunogenicity and antigenicity of PSA derived peptides in HhD mice. Procedures were performed and results are presented as in Figure 8.

FIG. 10 demonstrates immunogenicity and antigenicity of PSMA derived peptides in HhD mice. Procedures were performed and results are presented as in Figure 8.

FIG. 11 demonstrates lysis of PSA, PAP and PSMA derived peptide loaded RMA-S-HhD cells by CTL induced against DU145-HhD cells in HhD mice. Mice were immunized with irradiated DU145-HhD cells and CTL assays were performed as described in Materials and Methods. Labeled RMA-S-HhD target cells were loaded with synthetic PSA (1-5), PAP (1-4), PSM-A (1-6) or tyrosinase (RMA-S Tyr) peptides, with prostate carcinoma (RMA-S CAP), breast carcinoma (RMA-S Br.) or hyperplastic prostate (RMA-S HP) extracted peptides. DU145 (DU) and DU145-HhD also served as targets. The results are presented in lytic units as in Figure 4.

FIG. 12 demonstrates lysis of PSA, PAP and PSMA derived peptide loaded RMA-S-HhD by CTL induced against prostate carcinoma extracted peptides in HhD mice. HhD mice were immunized with RMA-S-HhD-B7.1 cells loaded with peptides extracted from prostate carcinoma samples. Experimental details are equivalent to the details in Figure 11.

FIG. 13 demonstrates stabilization of cell surface HhD on RMA-S- cells by human BA-46 (Lactadherin) derived peptides. RMA-S-HhD cells were incubated with 1-100  $\mu$ M synthetic peptides and monitored as described in Figure 1.

FIG. 14 demonstrates immunogenicity of BA-46 peptides in HhD mice. Mice were immunized as described in Materials and Methods, with RMA-S-HhD-B7.1 cells loaded with synthetic peptides. CTL assays were performed on RMA-S-HhD target cells loaded with homologous peptides.

FIG. 15 demonstrates lysis of BA-46 peptide loaded targets by CTL induced against breast carcinoma extracted peptides in HhD mice. HhD mice were immunized with RMA-S-HhD-B7.1 cells loaded with peptides extracted



from breast carcinoma samples. Experimental details are as in Figure 11. The data is presented as percent specific lysis at E:T of 100:1.

FIG. 16 demonstrates differential lysis of tumor extracted peptide loaded target cells by CTL induced against tumor peptides or BA-46 derived peptides in HhD mice. Mice were immunized by RMA-S-HhD-B7.1 cells loaded with synthetic BA-46 derived peptides or breast carcinoma derived peptides as described in Materials and Methods. CTL were tested against RMA-S-HhD cells loaded with breast carcinoma extracted peptides or normal breast tissue extracted peptides. Percent specific lysis at E:T of 100:1 is presented.

FIG. 17 demonstrates HLA-A2.1 (HhD) restricted lysis of a breast carcinoma cell line by CTL induced against BA-46 derived peptides or tumor extracted peptides. HhD mice were immunized as in Figure 16 and CTL were tested against the HLA negative MDA-MB-157 and their HhD transfectants. The data is presented in percent specific lysis at a E:T of 100:1.

FIG. 18 demonstrates stabilization of cell surface HhD on RMA-S- cells by Mucin (MUC-1/A7, E6, D6) derived peptides. Details are as described in Figure 1.

FIG. 19 demonstrates immunogenicity of MUC-1 derived peptides. HhD mice were immunized and CTL tested as in Figure 14.

FIG. 20 demonstrates HLA-A2.1 (HhD) restricted lysis of a breast carcinoma cell line by CTL induced against MUC-1 derived peptides. Details as described in Figure 17.

FIG. 21 demonstrates lysis of MUC-1 loaded target cells by CTL induced against breast carcinoma extracted peptides in HhD mice. Details are as described in Figure 15. E:T is 50:1.

FIG. 22 demonstrates differential lysis of tumor extracted peptide loaded target cells by CTL induced against MUC-1 derived peptides in HhD mice. Details are as described in Figure 16.

FIGs. 23a-e demonstrate lysis of additional human Uroplakin (Ib, II and III) peptide loaded target cells by CTL induced against the peptides in HhD mice. Mice were immunized with RMA-S-HhD-B7.1 cells, loaded with Uroplakin derived synthetic peptides, as described in Materials and Methods. Loading was performed with individual peptides and cells were pooled for immunization. Resensitized CTL (see Materials and Methods) were tested for lysis of RMA-S-HhD targets loaded with Uroplakin peptides or with an irrelevant HLA-A2 binding peptide derived from tyrosinase. Results are expressed as % Lysis using E:T ratios of 100:1, 50:1, 25:1, and 12.5:1. In Figs. 23a-d peptides were loaded onto target cells at a concentration of 200  $\mu$ M. For Fig. 23e two

concentrations of peptide were used for loading (20  $\mu$ M and 200  $\mu$ M). The protein origins of the peptides examined are listed in Table 3.

FIG. 24a-c demonstrate lysis of human CRIPTO-a peptide loaded target cells by CTL induced against peptides in HhD mice. Mice were immunized with RMA-S-HhD-B7.1 cells, loaded with CRIPTO-1 derived synthetic peptides, as described in Materials and Methods. Loading was performed with individual peptides and cells were pooled for immunization (pool of C1, C3, C5, and C7 for Fig. 24, pool of C2, C4, C6, and C8 for Fig. 24b, and pool of C10-12 for Fig. 24c). Resensitized CTL were tested for lysis of RMA-S-HhD targets loaded with CRIPTO-1 peptides at two concentrations (200 or 20  $\mu$ M), or with an irrelevant HLA-A2 binding peptide (C2 for Fig. 24a, C10 for Fig. 24b). Results are expressed a % Lysis using E:T ratios of 100:1, 50:1, 25:1, and 12.5:1.

#### 15 DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention concerns peptides and pharmaceutical and vaccine compositions including same which can be used to prevent or cure cancer, both primary tumors and metastases. Specifically, the present invention can be used to provide vaccines which include novel and potent tumor associated antigen peptides derived from Uroplakins, Prostate specific antigen, Prostate acid phosphatase, Prostate specific membrane antigen, Lactadherin Mucin and Teratocarcinoma-derived growth factor, which can be used as anti-tumor vaccines to prevent or cure, for example, bladder, prostate or breast cancers, carcinomas in particular, or any other tumor expressing the above listed proteins.

25 The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

35 A specific CTL response was found in patients with bladder transitional cell carcinoma (TCC) against the tumor extract and against peptides homologous to Uroplakin II sequence, which was significantly augmented after BCG intravesical therapy. All Uroplakin II peptides employed were good immunizers in HhD mice and CTL from these mice induced intense lysis of targets loaded

with Uroplakin II homologue peptides, TCC peptide extract and of the J82 HLA-A2 expressing cell line. CTL from HhD mice immunized with TCC peptide extract showed cross reactivity and induced significant lysis of targets loaded with Uroplakin II, or Uroplakin Ia homologue peptides, with TCC peptide extract and of J82 cells. For all immunized HhD mice, there was reduced lysis of targets loaded with normal bladder mucosa peptide extract and of TCC SUP cells (minimal HLA-A2 expression). Also there was no non-specific lysis. In addition, a cross reactivity of CTL induced by human Uroplakin II peptides against murine homologue peptides was found. Despite this finding there was no damage, or inflammatory infiltrate in the internal organs, including the urinary bladder on careful histological examination. These data indicate that Uroplakin homologue peptides constitute specific CTL epitopes and may be considered for immunotherapeutic vaccines alone, or in combination with intravesical BCG instillations in patients prone to recurrence and progression of bladder TCC. Similar results were obtained with human Uroplakin Ib and III.

By using HhD mice, HLA-A2 transgenic and which do not express murine MHC class I, it was found that 3 Prostate specific antigen (PSA), 4 Prostate specific membrane antigen (PSMA) and 4 Prostate acid phosphatase (PAP) homologue peptides were immunogenic. The 3 PSA homologue peptides and 2 of the 4 PSMA homologue peptides were not evaluated previously. The 2 PSMA homologue peptides, which were evaluated previously and were found to be CTL epitopes in CAP patients, were also found to be immunogenic in HhD mice. PAP homologue peptides were not evaluated previously and all 4 HLA-A2 binding peptides were very immunogenic in HhD mice and induced also, specific lysis of DU145-HhD cells. Moreover, CTL derived from HhD mice immunized with CAP peptide extract, or with DU145-HhD cells induced specific lysis of targets loaded with CAP peptide extract and PAP homologue peptides. There was also cross reactivity between CTL derived from CAP immunized HhD mice and PSA, or PSMA homologue peptide loaded targets. Low cross reactivity was found for CTL derived from DU145-HhD immunized HhD mice, probably due to low expression of these proteins by DU145 cells. There was no damage, or inflammatory infiltrate in the internal organs of immunized or control HhD mice on careful histological examination. These data indicate that PSA, PSMA and especially PAP homologue peptides constitute specific CTL epitopes and may be considered for immunotherapy of CAP patients.

It was further found a specific CTL response in HhD mice against breast tumor extract and against novel peptides complementary to Lactadherin (BA-46) sequences. These data indicate that BA-46 peptides constitute specific CTL

epitopes enriched in breast carcinoma and may be considered for immunotherapeutic vaccines.

It was yet further found that Mucin (MUC-1) derived peptides are potential TAA peptides that can be used in anti-tumor vaccine preparations. CTL induced  
5 by these peptides lyse better tumor extract loaded targets than normal tissue extract loaded targets. The lysis is HLA-A2 restricted and breast specific. Three novel peptides, from non-tandem repeat array (TRA) domains were shown to constitute potential CTL epitopes.

Thus, in accordance with one aspect of the teachings of the present  
10 invention there is provided a vaccine composition which includes at least one tumor associated antigen peptide derived from a protein selected from the group consisting of Uroplakin (UP), Prostate specific antigen (PSA), Prostate specific membrane antigen (PSMA), Prostate acid phosphatase (PAP), Lactadherin (BA-46) and Mucin (MUC1).

15 A specific CTL response has been obtained from Teratocarcinoma-derived growth factor (CRIPTO-1) derived peptide in HhD transgenic mice. This data indicates CRIPTO-1 peptides may constitute effective CTL epitopes in humans with HLA-A2 haplotypes and may be considered for immunotherapeutic cancer vaccines.

20 According to another aspect of the present invention there is provided a method of vaccination for prevention or cure of cancer. The method is effected by administering to a patient a vaccine composition including at least one tumor associated antigen peptide derived from a protein selected from the group consisting of Uroplakin (UP), Prostate specific antigen (PSA), Prostate specific  
25 membrane antigen (PSMA), Prostate acid phosphatase (PAP), Lactadherin (BA-46) and Mucin (MUC1).

Immunotherapy by *in vivo* DNA transfer of DNA coding for TAA is based on the rationale of quality or quantity increased peptide presentation leading to activation of an immune response against these peptides. Gene or DNA  
30 vaccination results in the intracellular processing and presentation of immunogenic peptides (99). Initial reports on DNA vaccination showed that "naked" DNA injected into the muscle tissue of a mouse is expressed efficiently (32). Embryonically expressed TAA such as CEA was tested (33). Immunization of mice with CEA expressing plasmid DNA was indeed found to protect 100 % of  
35 these mice against a challenge with CEA-expressing colon carcinoma cells (34). Both cellular and humoral responses have been reported after DNA vaccination in mice. In other studies a MUC-1 tandem repeat array was used for DNA vaccination of mice and 30% of these mice were protected from a tumor challenge

with MUC-1 transfected murine tumor cells (35). DNA vaccination may also be used to elicit immune responses against predefined peptide epitopes, several groups now exploit the string-bead approach to link multiple different CTL or helper epitopes together on the DNA level (36). In some cases the string-bead of peptide coding DNA is built into a vaccinia virus as a delivery vehicle. Recently, it was shown that such a vaccinia virus recombinant poly-epitope vaccine was able to protect mice against several virus infections and a tumor challenge (37). The authors show that all 10 minimal peptide epitopes encoded by the string-bead are expressed and recognized by the appropriate T cell clones (38). RNA was also shown to confer anti-tumor immunity. Vaccination with RNA to ovalbumin induced CTL in mice (39). In conclusion, multiple studies have shown the efficacy of DNA vaccines in anti-viral and anti-tumor immunity.

Thus, according to yet another aspect of the present invention there is provided a DNA vaccine composition which includes at least one polynucleotide encoding a tumor associated antigen peptide derived from a sequence encoding a protein selected from the group consisting of Uroplakin (UP), Prostate specific antigen (PSA), Prostate specific membrane antigen (PSMA), Prostate acid phosphatase (PAP), Lactadherin (BA-46), Mucin (MUC1) and Teratocarcinoma-derived growth factor (CRIPTO-1). As further detailed herein, the at least one polynucleotide can be a part of a longer polynucleotide designed to encode a fused protein product from which the tumor associated antigen peptide is cleavable by a protease.

The polynucleotide is preferably DNA in a form of, or contained in, for example, naked DNA, plasmid, retroviral vector, adenoviral vector, vaccinia viral vector, herpes viral vector, lenti virus vector, EBV vector, CMV vector, polio virus vector, sindbis viral vector, semliki forest virus vector, parvo virus vector, adeno-associated virus vector, virus like particle (VLP) vector. Alternatively, the polynucleotide can be in the form of RNA. The polynucleotide can also be delivered in a non-viral delivery system, such as, for example, but not limited to, in liposomes, in complex with cationic reagents, or with a polycation, such as poly-lysine. The polynucleotide can also be delivered by mechanical means, such as, but not limited to, a gene-gun, by electrical means, or in bacterial vectors like BCG.

There is increasing evidence that peptide vaccination may be much more effective when the peptides are introduced together with an antigen presenting cell (APC) (40). In previous studies of a murine lung carcinoma we have shown that vaccination with a defined TAA peptide (MUT-1) loaded on APC result in long term survival of mice bearing lung metastases (41, 42). The most common cells

used to load antigens are bone marrow and peripheral blood derived dendritic cells (DC), as these cells express costimulatory molecules that help activation of CTL. Preliminary clinical trials have been performed. In one trial HLA-A1 melanoma patients have been treated with autologous DC loaded with a MAGE-1 peptide. CTL activity was increased in tumor infiltrated lymphocytes (43). In another study, five patients with advanced pancreatic carcinoma were treated with a K-ras derived peptide loaded on DC. As a mutation of K-ras at codon 12 is frequently found in pancreatic carcinoma, three differently mutated peptides, 12-Asp, 12-Arg and 12-Val (non mutated sequence is 12-Gly) were used for vaccination, matched to the mutation in the patient's tumor. Two of the patients showed a specific CTL response and prolonged survival (44). A phase I clinical trial in 51 prostate cancer patients compared a soluble peptide to a DC based peptide in HLA-A2 patients. The peptide was derived from PSMA (SEQ ID NO:29). Only 7 patients that received DC based vaccines with this peptide responded by decreased levels of serum PSA (45). In animal studies a number of groups showed that macrophages loaded with peptides constitute efficient vaccines, yet the number of cells used for vaccination is 10 fold higher than equivalent DC vaccines. Recently in a murine lung carcinoma model the efficacy was tested of syngeneic fibroblasts treated with a proteasome inhibitor to decrease levels of endogenous peptides and loaded with synthetic MUT peptides as vaccines. Effective protection was found against metastatic spread of lung carcinoma.

Thus, according to still another aspect of the present invention there is provided a cellular vaccine composition which includes an antigen presenting cell presenting at least one tumor associated antigen peptide derived from a protein selected from the group consisting of Uroplakin (UP), Prostate specific antigen (PSA), Prostate specific membrane antigen (PSMA), Prostate acid phosphatase (PAP), Lactadherin (BA-46), Mucin (MUC1) and Teratocarcinoma-derived growth factor (CRIPTO-1). As further detailed herein, the antigen presenting cell can, for example, be a dendritic cell, a macrophage, a B cell and a fibroblast. Presenting the at least one tumor associated antigen peptide can be effected by a method selected from the group consisting of (a) transducing the antigen presenting cell with at least one polynucleotide (e.g., DNA) encoding the at least one tumor associated antigen peptide; (b) loading the antigen presenting cell with at least one polynucleotide (e.g., RNA) encoding the at least one tumor associated antigen peptide; (c) loading the antigen presenting cell with the at least one tumor associated antigen peptide (e.g., synthetic); and (d) loading the antigen presenting cell with at least one longer polypeptide (e.g., purified) including the at least one tumor associated antigen peptide. Loading can be external or internal. The

polynucleotide, peptide or longer polypeptide can be fused to internalizing sequences, antennapedia sequences or toxoid sequences or to helper sequences, such as, but not limited to, heat shock protein sequences.

While it is clear that CD8+ class-I restricted CTL recognize and destroy tumor cells in vitro and in vivo, animal models often show a requirement of CD4+ MHC-class-II restricted T cell help for optimal responses (83). Helper T cell epitopes can contribute to induction of cellular immune responses by class I peptide vaccines, as seen by the synergistic tumor protection upon simultaneous vaccination with T helper and CTL epitopes (84). The 'help' to CTL is most often provided via the production of specific cytokines. Helper epitopes can be specific and derived from a tumor antigen (85). They can also broadly crossreact with a number of MHC class II molecules, and may be either pathogen-derived or comprised of sequences not found in nature (86-88). More specifically, a sequence containing a T helper epitope can be linked to a CTL epitope to create one immunogenic entity. Alternatively, a mixture of 2 or more separate entities, corresponding to CTL and T helper epitopes can be administered to elicit the desired CTL response. T helper epitopes can also be conjugated to other molecules or compounds which increase their biological activity.

As used herein in the specification and in the claims section below the phrase "tumor associated antigen" also refers to tumor specific antigen.

As used herein in the specification and in the claims section below the term "peptide" refers to native peptides (either degradation products or synthetically synthesized peptides) and further to peptidomimetics, such as peptoids and semipeptoids which are peptide analogs, which may have, for example, modifications rendering the peptides more stable while in a body, or more immunogenic. Such modifications include, but are not limited to, cyclization, N terminus modification, C terminus modification, peptide bond modification, including, but not limited to, CH<sub>2</sub>-NH, CH<sub>2</sub>-S, CH<sub>2</sub>-S=O, O=C-NH, CH<sub>2</sub>-O, CH<sub>2</sub>-CH<sub>2</sub>, S=C-NH, CH=CH or CF=CH, backbone modification and residue modification. Methods for preparing peptidomimetic compounds are well known in the art and are specified in Quantitative Drug Design, C.A. Ramsden Gd., Chapter 17.2, F. Choplin Pergamon Press (1992), which is incorporated by reference as if fully set forth herein. Further detail in this respect is provided herein.

As used herein in the specification and in the claims section below the term "amino acid" is understood to include the 20 naturally occurring amino acids; those amino acids often modified post-translationally *in vivo*, including for example hydroxyproline, phosphoserine and phosphothreonine; and other unusual

amino acids including, but not limited to, 2-amino adipic acid, hydroxylysine, isodesmosine, nor-valine, nor-leucine and ornithine. Furthermore, the term "amino acid" includes both D- and L-amino acids. Further elaboration of the possible amino acids usable according to the present invention and examples of non-natural amino acids useful in MHC-1 recognizable peptide antigens are given herein.

Peptides can be of 8, 9 or 10 amino acids and peptides of 9 or 10 amino acid residues may be desirable, preferably 9. Thus, assume the following positions (P1-P9) in a 9-mer peptide:

10 P1-P2-P3-P4-P5-P6-P7-P8-P9

The P2 and P9 positions include the anchor residues which are the main residues participating in binding to MHC class 1 molecules, more specifically HLA-A2. Amino acid residues engaging positions P2 and P9 are hydrophobic or hydrophilic natural amino acids or non-natural amino acids. Examples of natural amino acids being Ala, Cys, Gln, Glu, Ile, Leu, Met, Ser, Thr and Val. These residues may preferably be neutral, hydrophobic, aliphatic and more preferably Val, Leu and Ile. Examples of non-natural amino acids being norleucine (Nle), norvaline (Nva), aminobutyric acid preferably  $\alpha$ -aminobutyric acid. These residues may preferably be non charged and more preferably aliphatic. P9 can also be an aliphatic amino acid of the general formula  $\text{-HN(CH}_2\text{)}_n\text{COOH}$ , wherein  $n = 2-5$ , as well as by branched derivatives thereof, such as, but not limited to,



wherein R is, for example, methyl, ethyl or propyl, located at any one or more of the n carbons.

Positions P1 and P3 are also known to include amino acid residues which participate or assist in binding to MHC molecules, however, these positions can include any amino acids, natural or non-natural.

The amino terminal residue (position P1) can also be positively charged aliphatic carboxylic acids, such as, but not limited to,  $\text{H}_2\text{N(CH}_2\text{)}_n\text{COOH}$ , wherein  $n = 2-5$  and  $\text{H}_2\text{N-C(=NH)-NH(CH}_2\text{)}_n\text{COOH}$ , wherein  $n = 2-4$ , hydroxy Lysine,  $\text{N}^\epsilon$ -methyl Lysine,  $\text{N}^\epsilon$ -ethyl Lysine,  $\text{N}^\epsilon$ -propyl Lysine or ornithine (Orn). Additionally, the amino terminal residue can be aromatic residues, such as, but not limited to, phenyl glycine, p-aminophenyl alanine, p-guanidinophenyl alanine or pyridinoalanine (Pal). These latter residues may form hydrogen bonding with the



OH<sup>-</sup> moieties of the Tyrosine residues at the MHC-1 N-terminal binding pocket, as well as to create, at the same time aromatic-aromatic interactions

The other positions P4-P8 are engaged by amino acid residues which typically do not participate in binding to MHC molecules, rather these amino acids are presented to the immune cells. Further details relating to the binding of peptides to MHC molecules can be found in reference 82, see Table V thereof, in particular.

Amino acid residues engaging position P4-P8 can include any amino acids natural or non natural. These residues may optionally be phosphorylated and/or glycosylated. Indeed residues which have been phosphorylated or glycosylated have been shown in some cases to enhance the binding to the T cell receptor.

Cyclization can engage position P4-P8, preferably positions P6 and P7. Cyclization can be obtained through amide bond formation, e.g., by incorporating Glu, Asp, Lys, Orn, di-amino butyric (Dab) acid, di-aminopropionic (Dap) acid at various positions in the chain (-CO-NH or -NH-CO bonds). Backbone to backbone cyclization can also be obtained through incorporation of modified amino acids of the formulas  $H-N((CH_2)_n-COOH)-C(R)H-COOH$  or  $H-N((CH_2)_n-NH_2)-C(R)H-COOH$ , wherein  $n = 1-4$ , and further wherein R is any natural or non-natural side chain of an amino acid.

Cyclization via formation of S-S bonds through incorporation of two Cys residues is also possible. Additional side-chain to side chain cyclization can be obtained via formation of an interaction bond of the formula  $-(CH_2)_n-S-CH_2-C(=O)-$ , wherein  $n = 1$  or  $2$ , which is possible, for example, through incorporation of Cys or homoCys and reaction of its free SH group with, e.g., bromoacetylated Lys, Orn, Dab or Dap.

In longer peptides such as in a 10 mer peptide in which the second anchor amino acid is at position P10 the amino acid engaging P9 may include most L amino acids. In some cases shorter peptides such as an 8 mer peptide are also applicable, in which the carboxy terminal acid P8 may serve as the second anchor residue. All the options described for the anchor amino acid residues engaging positions P2 and P9 in a 9 mer peptide may apply likewise to the anchor amino acid residues engaging positions P2 and P10 in a 10 mer peptide and P2 and P8 in an 8 mer peptide.

The amino acids may be modified as is necessary to provide certain characteristics such as greater immunogenicity, more stability or improved pharmacological properties. The peptides can be for instance subject to changes such as the replacement of one or more amino acid residues whether dissimilar or similar.

Modification of the peptides may also be by decreasing, e.g. in a 10 mer peptide, or extending, e.g. in an 8 mer peptide, the amino acid sequence, for example, by deletion or addition of amino acids. It will be appreciated that preferably anchor amino acids should not be deleted.

5 Peptide bonds (-CO-NH-) within the peptide may be replaced by N-alkylated bonds such as N-methylated (-N(CH<sub>3</sub>)-CO-), ester bonds (-C(R)H-C-O-O-CH(R)-N-), ketomethylen bonds (-CO-CH<sub>2</sub>-), -aza bonds (-NH-N(R)-CO-), wherein R is hydrogen or any alkyl, e.g., methyl carba bonds (-CH<sub>2</sub>-NH-), hydroxyethylene bonds (-CH(OH)-CH<sub>2</sub>-), thioamide bonds  
10 (-CS-NH-), olefinic double bonds (-CH=CH-), retro amide bonds (-NH-CO-), and peptide derivatives (-N(R)-CH<sub>2</sub>-CO-), naturally presented on the carbon atom.

These modifications can occur at any of the bonds along the peptide chain and even at several (2-3) at the same time. Preferably, but not in all cases necessary, these modifications should exclude anchor amino acids.

15 For amino acid residues engaging positions other than the second residue from the amino terminal and the end residue at the carboxy terminal natural aromatic amino acids, Trp, Tyr and Phe, may be replaced by synthetic non-natural acid such as TIC, naphthylalanine (Nal), ring-methylated derivatives of Phe, halogenated derivatives of Phe or o-methyl-Tyr.

20 As used herein in the specification and in the claims section below, the term "transduced" refers to the result of a process of inserting nucleic acids into cells. The insertion may, for example, be effected by transformation, viral infection, injection, transfection, gene bombardment, electroporation or any other means effective in introducing nucleic acids into cells. Following transduction the  
25 nucleic acid is either integrated in all or part, to the cell's genome (DNA), or remains external to the cell's genome, thereby providing stably transduced or transiently transduced cells.

As used herein in the specification and in the claims section below the phrase "derived from a protein" refers to peptides derived from the specified  
30 protein or proteins and further to homologous peptides derived from equivalent regions of proteins homologous to the specified proteins of the same or other species, provided that these peptides are effective as anti-tumor vaccines. The term further relates to permissible amino acid alterations and peptidomimetics designed based on the amino acid sequence of the specified proteins or their  
35 homologous proteins.

As used herein in the specification and in the claims section below the phrase "anti-tumor vaccines" refers to vaccines effective in preventing the development of, or curing, cancer, including primary tumor and/or metastases.

As used herein in the specification and in the claims section below the phrase "prevention or cure" also refers to inhibiting, slowing or reversing the progression of a disease, substantially ameliorating clinical symptoms of a disease or substantially preventing the appearance of clinical symptoms of a disease.

5 In the specification and in the claims section below the phrase as used herein 'loading' refers to exposing, adding or introducing a substance into or onto a cell or vesicle or part thereof.

According to a preferred embodiment of the present invention the Uroplakin (UP), Prostate specific antigen (PSA), Prostate specific membrane  
10 antigen (PSMA), Prostate acid phosphatase (PAP), Lactadherin (BA-46), Mucin (MUC1) and Teratocarcinoma-derived growth factor (CRIPTO-1) proteins are each independently of a mammal, e.g., a humanoid such as a human being or a rodent such as murine.

According to yet another preferred embodiment of the present invention the  
15 composition further comprising a carrier. Usually the tumor associated antigen peptide(s) are presented in context of the carrier.

The carrier can be a proteinaceous carrier to which the peptides are linked. Methods of linking short peptides to carriers are well known in the art of vaccination. The carrier can alternatively be a particulate adjuvant, an oil or  
20 emulsifier based adjuvant, a gel based type adjuvant, or an adjuvant based on specific targeting of antigen, such as, but not limited to, antibody-liposom conjugates. The carrier can also be a protein or a recombinant protein produced, for example in bacteria, yeast or in mammalian cells, including cytokines, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13,  
25 IL-14, IL-15, IL-16, IL-17, IL-18, interferon alpha, interferon beta, interferon gamma and others. The carrier can also be an antigen presenting cell, such as, but not limited to, a dendritic cell, a macrophage, a B cell or a fibroblast. The cell selected is either an autologous or non-autologous HLA matching cell. Optionally the cell can be a cultured cell, a cell treated by various reagents (e.g., by early  
30 and/or late acting cytokines), transduced by genes, and/or irradiated or radiated.

The vaccine composition according to the present invention is effective in prevention or cure of cancer and/or cancer metastases. In other words, the composition is effective for primary tumors, secondary tumors and metastases thereof in the same organ or in another organ, provided that the tumor expresses  
35 the above listed tumor associated proteins. According to a preferred embodiment of the present invention the cancer being treated or prevented via the administration of the vaccine composition is a carcinoma. i.e., a malignant tumor composed of epithelial tissue. The cancer treated or prevented according to the

present invention can be, for example, breast, bladder, prostate, pancreas, ovary, thyroid, melanoma, colon, stomach and/or head and neck cancer.

According to an embodiment of the present invention, the Uroplakin protein can be Uroplakin II, Uroplakin Ia, Uroplakin III and Uroplakin Ib.

5 According to another embodiment of the present invention, the tumor associated antigen peptides derived from the Mucin protein are from a non-tandem repeat array of Mucin.

According to yet another embodiment of the present invention, one or more tumor associated antigen peptides derived from the Mucin protein are from a  
10 region selected from the group consisting of a signal peptide, a cytoplasmic domain and an extracellular domain of Mucin.

According to a presently preferred embodiment of the present invention the one or more tumor associated antigen peptides are selected from SEQ ID NOs: 1 to 64 and 66 to 77 and effective homologues and analogs thereof.

15 For therapeutic or prophylactic anti-tumor treatment, the vaccine composition according to the present invention may include thickeners, carriers, buffers, diluents, surface active agents, preservatives, and the like, all as well known in the art. The composition may also include one or more active ingredients, such as, but not limited to, anti-inflammatory agents, anti-microbial  
20 agents, anesthetics and the like.

The vaccine composition may be administered in either one or more ways. Administration may be effected topically (including ophthalmically, vaginally, rectally, intranasally), orally, by inhalation, or parenterally, for example by intravenous drip or intraperitoneal, intravesical, subcutaneous, or intramuscular  
25 injection.

Compositions for topical administration can include, but are not limited to, lotions, ointments, gels, creams, suppositories, drops, liquids, sprays and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

30 Formulations for parenteral administration can include, but are not limited to, sterile aqueous solutions which may also contain buffers, diluents, adjuvant and other suitable additives. The adjuvant is preferably of a type allowed for use in treating human beings, such as BCG adjuvant.

Dosing is dependent on responsiveness, but will normally be one or more  
35 doses per week or month, with course of treatment lasting from several weeks to several months. Persons ordinarily skilled in the art can easily determine optimum dosages, dosing methodologies and repetition rates.

The present invention provides novel tumor associated antigen peptides effective in eliciting CTL response which can therefore be effective therapeutic agents to combat cancer.

According to another embodiment of the present invention there is provided a peptide derived from a protein selected from the group consisting of Uroplakin (UP), Prostate specific antigen (PSA), Prostate specific membrane antigen (PSMA), Prostate acid phosphatase (PAP), Lactadherin (BA-46) and Mucin (MUC1) and Teratocarcinoma-derived growth factor (CRIPTO-1), the peptide comprising 8-10 amino acid residues as selected so as to promote effective binding to a MHC class 1 type molecule such that a CTL response is elicitable

Each of the various aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the Examples section that follows.

15

## EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

20

## MATERIALS AND EXPERIMENTAL METHODS

**Mice:** The derivation of HLA-A2.1/D<sup>b</sup>- $\beta$ 2m monochain, transgenics, H-2D<sup>b</sup> x  $\beta$ 2m double knockout mice (HhD mice) was described before (30).

**Tumor and cell lines:** RMA-S is a TAP-2 deficient lymphoma clone of C57BL/6 origin. The RMA-S HhD and RMA-S-HhD-B7.1 are transfectants carrying the HhD construct without or with the gene for murine B7-1 costimulatory molecule, respectively. T2 is a TAP-2 deficient human lymphoblastoid line expressing HLA-A2. RMA-S, RMA-S derivatives and T2 cells were maintained in RPMI 1640-10 % FCS and antibiotics.

30

The human tumor cell lines, J82 (TCC of the bladder) sup (TCC of the bladder, DU145 (prostate carcinoma) and MDA-MB-157 (breast carcinoma) and the HhD transfectants of DU145 and MDA-MB-157 were maintained in DMEM-10 % FCS 2 mM glutamine - 1 mM sodium pyruvate - 1 % non-essential amino acids and antibiotics.

35

HhD and B7-1 transfectants were grown in presence of 500-1000  $\mu$ g/ml of Genitcin, G418. All tissue culture media and supplements were purchased from Gibco.

**Peptide synthesis:** Peptides were synthesized on an ABIMED AMS 422 multiple peptide synthesizer (Langenfeld, Germany), employing the a-N-9-fluorenylmethoxycarbonyl (Fmoc) strategy following the company's commercially available protocols. Peptide chain assembly was conducted on a 2-chlorotrityl chloride resin (Novabiochem, Laufelfingen, Switzerland). Crude peptides were purified to homogeneity by reversed-phase HPLC on a semi-preparative silica C-8 column (250 x 10 mm; Lichnonorb RP-8; Merck). Elution was accomplished by a linear gradient established between 0.1 % trifluoroacetic acid in water and 0.1 % trifluoroacetic acid in 70 % acetonitrile in water (v/v). Purity of peptides ( 96 %) was ascertained by analytical HPLC (RP-18) using various acetonitrile water (containing 0.1 % trifluoroacetic acid) gradients. The products' compositions were determined by amino acid analysis (Dionex automatic amino acid analyzer, Sunnyvale, CA) after extraction acid hydrolysis. Molecular weight was ascertained by mass spectrometry (VG Tofspec; Laser Desorption Mass. Spectrometry; Fison Instruments, Manchester, U.K.). Tables 1-8 present these peptides.

**Preparation of peptide fractions from fresh human tumors and normal tissues:** Surgical specimens of TCC, normal bladder mucosa, prostate carcinoma, hyperplastic benign prostate, breast carcinoma or normal breast tissue were transferred on ice from surgery to a qualified pathologist. Tumor tissue were cleaned from most adjacent tissue and snap frozen. Non-necrotic fragments, from 5-10 patients were homogenized in PBS; 0.5 % Nonidet P-40; 10 µg/ml soybean trypsin inhibitor; 5 µg/ml lupeptide; 8 µg/ml aprotinin and 0.5 mM PMSF and homogenized using a glass-teflon homogenizer. Following further stirring for 30 minutes at 4°C, the homogenates were titrated with 10 % TFA to a final concentration of 0.1 % TFA and stirred for 30 minutes at 4°C. After ultra-centrifugation for 30 minutes at 42K rpm the supernatants were applied to Sephadex G25 columns and fractions were monitored at 230 nm. Peptide fractions under 10 Kd were pooled, lyophilized and further fractionated by centriprep 3 by centrifugation (Amicon, Beverly, MA). The peptide pool after lyophilization, was dissolved in opti-MEM (Gibco) for further use.

**Scoring of HLA-A2.1 binding peptides:** Protein sequences were screened for MHC binding by a HLA Peptide Binding Predictions software approachable through a worldwide web interface at (see also reference 82). This software, based on accumulated data, scores every possible peptide in the protein for possible binding to MHC according to the contribution of every amino acid in the peptide. Theoretical binding scores represent calculated half-life of the HLA-A2.1-peptide complex.

**Measurement of peptide binding by stabilization of cell surface MHC:**

Peptide binding to HhD was measured by stabilization of HhD on RMA-S transfectants, using an indirect FACS assay as follows:  $5 \times 10^5$  peptide loaded TAP-2 deficient RMA-S-HhD cells (see vaccination), were incubated with anti  
5 HLA monoclonal antibodies for 30 minutes at 4°C. After washing the cells with PBS - 0.5 %, BSA + 0.1 %, sodium azide, the second antibody, goat anti mouse-FITC (Jackson Lab.), was applied for 30 minutes at 4°C. Following washing, the amount of bound antibodies was detected by FACSScan (Bacson).

Mouse monoclonal antibodies B-9-12, w6/32 (anti HLA A, B, C) and  
10 B.B7.2 (anti HLA-A2.1) were used for analyses.

**Vaccination:** Mice were immunized intraperitoneally three times at 7-day intervals, with  $2 \times 10^6$  irradiated (5,000 rad) tumor cells, or with peptide-loaded RMA-S-HhD-B7.1 transfectants. Peptide loading of RMA-S-HhD-B7.1 cells was performed as follows: After washing the cells 3 times in PBS, the surface  
15 expression of HhD monochins was stabilized by a 4 hours culture at 26°C. Synthetic peptides, or peptide extracts were added to  $10 \times 10^6$  cells in 1 ml of opti-MEM (Gibco) medium to a concentration of 100  $\mu$ M or 1 mM, respectively. The cells were incubated at 26°C and for additional 3 hours at 37°C. Peptide loaded RMA-S-HhD $\sigma$  -  $\sigma$ B7.1 cells were irradiated (5000 rad), washed,  
20 resuspended in PBS and injected into mice. In mixed synthetic vaccines, RMA-S-HhD-B7.1 cells were loaded separately with each peptide and pooled before vaccination.

**In vitro cytotoxicity assays:** Mice were immunized intraperitoneally three times at 7-day intervals, with  $2 \times 10^6$  irradiated (5,000 rad) tumor cells, or with  
25 peptide-loaded RMA-S-HhD-B7.1 transfectants. Spleens were removed on day 10 after the last immunization and splenocytes were restimulated *in vitro* with either irradiated tumor cells (for mice immunized with tumor cells) or with lymphocytes pulsed with 100  $\mu$ M synthetic peptides or 1 mM patients-derived extract in opti-MEM (Gibco) medium. Restimulated lymphocytes were  
30 maintained in RPMI 1640 medium containing 10 % FCS, 1 mM glutamine, combined antibiotics, 1 mM sodium pyruvate, 10 mM HEPES, pH 7.4,  $5 \times 10^{-5}$  M  $\beta$ -mercaptoethanol, and 1 % nonessential amino acids (RPMI-HEPES medium) for 5 days. Viable lymphocytes (effector cells), were separated by lymphocyte-M (Cedarlane, Ontario, Canada) centrifugation, resuspended in RPMI-HEPES  
35 medium, and admixed at different ratios with  $5 \times 10^3$  peptide loaded RMA-S-HhD cells, previously loaded with different peptides (see vaccination). CTL assays were performed in U-shaped microtiter wells, at 37°C, 5 % CO<sub>2</sub> for 5 hours. Cultures were terminated by centrifugation at 1,000 rpm for 10 minutes at 4°C. A

total of 100  $\mu$ l of the supernatants was mixed with scintillation fluids and counted in a beta counter. Percentage of specific lysis was calculated as follows: % lysis = (cpm in experimental well - cpm spontaneous release)/(cpm maximal release - cpm spontaneous release) x 100. Spontaneous release was determined by  
5 incubation of 100  $\mu$ l-labeled target cells with 100  $\mu$ l of medium. Maximal release was determined by solubilization of target cells in 100  $\mu$ l 0.1 M NaOH.

**Sensitization of human PBL *in vitro*:** Patients were HLA typed by the Tissue Typing Unit of The Rabin Medical Center, Israel. Only HLA-A2.1 carrying haplotypes were used herein. Heparanized blood, 40 ml/patient was  
10 separated on Ficoll gradients, mononuclear cells were collected at the interphase, washed three times in PBS and resuspended in PBS. A third of the lymphocyte volume was incubated with tumor extracted peptides, at a concentration of 1 O.D230/ml for 2 hours at 37°C. The other two thirds of the cells were suspended at  $10^6$  cells/ml in RPMI - 10 % FCS, 2 mM glutamine, 1 mM sodium pyruvate, 10  
15 mM Hepes,  $5 \times 10^{-3}$  M  $\beta$ -mercaptoethanol and combined antibiotics. Peptide loaded cells were added together with 5-20 U/ml of recombinant human IL-2 (Peptotec, Rehovot, Israel). Cells were incubated for 5-7 days at 37 °C, 5 % CO<sub>2</sub> and then used in CTL assays as described above.

20

## EXPERIMENTAL RESULTS

### EXAMPLE 1

#### Uroplakins are T cell defined TAAs in transitional cell carcinoma (TCC)

25 Transitional cell carcinoma (TCC) of the bladder represents a prevalent cancer. Its incidence increases gradually during life and peaks in the 7th and 8th decade to 200/100,000. About 95 % of bladder tumors are TCC. Of these, 75 % are superficial papillary tumors and have a 15 % - 20 % progression rate during the first two years of follow up. Approximately, 20 % - 25 % of TCC present as  
30 muscle invasive tumors with aggressive behavior and a 40 % survival rate at 5 years (46). After surgical resection of superficial tumors, intravesical instillations of chemotherapeutics as Tiotepa or Mitomycin, or non-specific intravesical immunotherapy with Bacillus Calmette Guerein (BCG), reduce the frequency of recurrences and have a minimal effect on progression. BCG intravesical  
35 instillation was found to be the most effective in reducing the recurrence and progression rate of high grade papillary TCC and of transitional cell carcinoma *in situ*. The mechanism of action was not yet elucidated. However, following therapy there is a mononuclear infiltrate of the lamina propria of the bladder



mucosa. This infiltrate is rich in T-cells, macrophages and Langerhans cells (47). It was found that CD8 T-cells were activated after BCG intravesical therapy and induced specific lysis of TCC cells (48). Also, BCG intravesical instillation resulted in increased presentation of MHC complexes on transitional cell membrane (49). Lately, CTL, which induced specific lysis of autologous tumor cells, were identified among tumor infiltrating lymphocytes grown from transitional cell carcinomas of the bladder (50). It seems that specific cytotoxic T-cell immunity against TCC may be augmented by BCG intravesical therapy.

An important group of TAAs are actually differentiation antigens. Such antigens were shown to induce CTL in melanoma and in colon carcinoma patients (22, 23).

Transitional epithelium is a differentiated multilayered epithelium which is restricted to the urinary tract. It is composed of 4 to 7 cell layers: a basal cell layer; an intermediate 2-3 cell layer and an apical (luminal) layer composed of umbrella like cells. These latter cells are connected by tight junctions and create an impermeable barrier to water and urinary solutes.

Lately, differentiation proteins of transitional epithelium have been characterized morphologically and biochemically. These proteins were found in urothelial plaques which are present in the luminal plasma membrane of urothelial superficial (umbrella) cells. The molecular constituents of these plaques comprise four transmembranal proteins designated Uroplakins (UP); UP Ia (27 Kd); UP Ib (28 Kd); UP II (15 Kd) and UP III (47 Kd glycoprotein; the size of the core protein is 28.9 Kd). Uroplakin III probably plays a role in the formation of the urothelial glycocalyx and may interact with the cytoskeleton. An extensive evaluation of UP tissue distribution showed that UP are restricted to normal transitional cell epithelium and to TCC (51). In normal transitional epithelium the UPs were found in the apical membrane of the superficial umbrella cells. In superficial papillary TCC, the UPs were localized in the apical membrane of the luminal cells and in the cell membranes lining intra and inter-cellular lumina. In invasive TCC, UPs were localized more randomly in the cell membrane of the cells which infiltrated the stroma. Recently, the sequence of human Uroplakin II cDNA and Uroplakin Ia genomic sequence were determined and their protein sequences were deduced (NCBI accession Nos. 2190407 and 2098577, respectively). Since TCC patients treated by BCG were shown to have increased T cell infiltrates in the bladder mucosa, we first tested whether BCG treated patients show increased CTL activity against TCC derived peptides and whether UP II peptides may constitute CTL specific epitopes. We further used the TCC

model to test the T cell repertoire overlap between patient derived CTL and CTL induced in the unique H-2D<sup>b</sup><sup>-/-</sup> x  $\beta$ 2m<sup>-/-</sup> x HhD<sup>+/+</sup> (HhD) mice.

**Cytotoxic activity of human peripheral blood lymphocytes (PBL) against target cells pulsed with Uroplakin II peptide homologues:** The human UP II amino acid sequence was screened for potential HLA-A2 binding, 9 amino acids peptides by a HLA binding motif program. We chose that particular HLA allele because it is present in 45 % of the population. Seven 9-mer peptides, which were predicted to bind with high affinity to HLA-A2 were selected and synthesized (Table 1). The binding of these peptides to HLA-A2 was tested by an MHC stabilization assay utilizing TAP negative cells, which can present exogeneously bound peptides. The RMA-S cells (30) transfected by the HhD construct were loaded with various concentrations of UP II peptides and then reacted with anti-HLA antibody. High affinity binding was shown for all 7 peptides by FACS analyses (Figure 1).

TABLE 1

*Predicted human Uroplakin II peptides that bind to HLA-A2*

Peptide	Sequence	Amino-acids*	SEQ ID:
HURO1	FLLVLGFII	168-176	1
HURO2	VLPSVAMFL	161-169	2
HURO3	LVLGFIIAL	170-178	3
HURO4	KVVTSSFVV	67-77	4
HURO5	LVPGTKFYI	109-117	5
HURO6	LLPIRTLPL	4-12	6
HURO7	YLVKKGTAT	119-127	7

The prediction program is described in Materials and Methods. The peptides are listed in descending order of predicted HLA-A2.1-peptide complex stability. Single letter amino acid codes and the position of the peptide in the protein are listed. \* Numbering is according to NCBI accession No. 2190407.

To restimulate CTL activity in patient's PBL we chose 6 TCC patients of the HLA-A2 haplotypes: 4 patients underwent adjuvant intravesical BCG instillations and 2 underwent no adjuvant therapy after endoscopic resection of the TCC. Another patient without TCC served as control. PBL were stimulated *in vitro* by a mixture of tumor acid extracted peptides from 9 TCC samples as described before (52). Each tumor was extracted individually and an equimolar

ratio of each sample was added to the mixture. The final concentration of peptides in the stimulation reaction was 1 O.D. 230/ml. Antigen presenting cells (APC) from the same peripheral blood were pulsed with the tumor extract. Peripheral blood lymphocytes (PBL) were stimulated with APC loaded with pooled TCC extract and with IL2 for 5 days. Uroplakin II peptides, TCC extracted peptides and normal bladder extracted peptides were loaded on labeled T2 cells (HLA-A2 positive cells which are TAP deficient, express empty MHC molecules and can be loaded only with exogenous peptides), and were screened by CTL obtained from PBL of the patients, as described. CTL from TCC patients treated with BCG induced 32 % to 71 % specific lysis of Uroplakin II peptide loaded T2 cells (Figure 2). Tumor extract loaded targets were lysed at 62% while normal bladder extract loaded targets were lysed at 29 % only, indicating preferential recognition of tumor antigens. CTL from TCC patients without BCG adjuvant therapy induced 25 % to 48 % specific lysis of T2 cells loaded Uroplakin II peptides, 29 % lysis of tumor extract loaded T2 cells and 15 % lysis of bladder extract loaded T2 cells. There was no lysis of T2 cells loaded with breast tumor extract, or with a HLA-A2 binding peptide homologue to tyrosinase, a melanoma differentiation antigen. A TCC cell line expressing HLA-A2, J82 was effectively killed while another TCC line SUP, was not lysed. Also, there was no lysis of a natural killer (NK) sensitive cell line (K562). CTL obtained from the patient without TCC induced no specific lysis of tumor extract, or Uroplakin II peptides loaded APC cells. The differences between TCC patients treated with BCG, TCC patients without adjuvant therapy and non-TCC patients were very significant ( $P < 0.001$ ). We also tested the activity of patient's PBL, resensitized by the pool of tumor extracts, against individual tumor extracts. Figure 3 shows higher activity of lymphocytes derived from BCG treated patients than lymphocytes derived from non-treated patients. Control lymphocytes show low activity. Thus shared antigens are expressed in all TCC samples.

**CTL induced in HhD mice recognize the same UP II peptides as human PBL:** The immunogenicity of Uroplakin homologue peptides was also evaluated in a mouse model, in which the murine MHC class I genes are not expressed ( $D^b^{-/-}$ ,  $\beta 2$ -microglobulin  $-/-$ , double knockout) and which is transgenic for a HLA-A2 and human  $\beta 2$ -microglobulin monochain (HhD mice). The advantage of a HLA-A2 transgenic mouse model is that CTL epitopes can be detected more easily and reproducibly, without lengthy and repeated *in vitro* stimulations. The murine MHC knockout mice used herein has the additional advantage that their CTL repertoire is only HLA-A2 restricted and the CTL response against human tumor extract or human cell lines is not masked by a

potential xenogeneic response. HhD mice were immunized with the 7 Uroplakin II homologue peptides or with the same TCC peptide extract used for stimulation of patient's PBL. Lymphocytes were obtained from the spleens of these mice and restimulated *in vitro* once with the corresponding peptides.

- 5 The 7 UP II peptides, that were recognized by human CTL, 8 new UP Ia homologues peptides with high HLA-A2 binding affinity (Table 2), 4 murine UP II derived HLA-A2 binding peptides (Table 3), TCC and normal bladder mucosa peptide extracts and two TCC cell lines: J82 (HLA-A2 positive) and TCCSUP were screened by the CTL obtained from these mice.

10

TABLE 2

*Predicted human Uroplakin Ia peptides that bind to HLA-A2*

Peptide	Sequence	Amino-acids*	SEQ ID:
HURO11	SLFAETIWV	33-41	8
15 HURO12	MLIAMYFYT	248-256	9
HURO13	LMWTLPVML	241-249	10
HURO14	MLIVYIFEC	100-108	11
HURO15	YIFECASCI	104-112	12
HURO16	LVLMLIVYI	97-105	13
20 HURO17	ALCRRRSMV	85-93	14
HURO18	LLSGLSLFA	28-36	15

The prediction program is described in Materials and Methods. The peptides are listed in descending order of predicted HLA-A2.1-peptide complex stability. Single letter amino acid codes and the position of the peptide in the protein are listed. \* Numbering is according to NCBI accession No. 2098577.

25

TABLE 3

*Predicted murine Uroplakin II peptides that bind to HLA-A2*

Peptide	Sequence	Amino-acids*	SEQ ID:
30 MURO1	FLLVVGLIV	168-176	16
MURO3	LVVGLIVAL	170-178	17
MURO4	KVVKSDFVV	69-77	18
MURO6	TLPVQTLPL	4-12	19

The prediction program is described in Materials and Methods. The peptides are listed in descending order of predicted HLA-A2.1-peptide complex stability. Single letter amino acid codes and the position of the peptide in the protein are listed. The murine 1, 3, 4, 6 peptides are homologous to the human Uroplakin II

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peptides 1, 3, 4, 6 in Table 1. \* Numbering is according to NCBI accession No. 586160.

The results are expressed as lytic units (LU30), which represent 10<sup>6</sup>/the  
5 number of effectors which induce 30 % specific target lysis. More LU signify a more potent CTL response. Figure 4 shows that CTL from HhD mice immunized with Uroplakin II peptides (1-7 loaded individually and injected by a mixture of loaded cells) induced a significant lysis of RMA-S-HhD cells loaded with Uroplakin II homologue peptides (8.1 to 32.2 LU). There was no lysis of  
10 RMA-S-HhD cells loaded with nonspecific peptides as breast tumor extract or the HLA-A2 binding tyrosinase homologue peptide. Also, there was no lysis of RMA-S-HhD cells without peptides (empty target cells). There was intense lysis of J82 cells (17.3 LU) and there was much less lysis of TCCSUP cells (1.2 LU). There was also intense lysis of RMA-S-HhD cells loaded with TCC peptide  
15 extract (15 LU) and less lysis of normal bladder mucosa peptide extract loaded cells (4.5 LU) (Figure 4).

CTL from mice immunized with TCC peptide extract induced significant lysis of RMA-S-HhD targets loaded with Uroplakin II homologue peptides (3.2 to 14 LU); Uroplakin Ia homologue peptides (2 to 11.4 LU); TCC peptide extract  
20 (12.6 LU) and less lysis of these targets loaded with normal bladder mucosa peptide extract (2 LU). There was also intense lysis of J82 cells (7.6 LU) and less lysis of TCCSUP cells (0.8 LU). There was no lysis of target cells loaded with nonspecific peptides or of empty target cells. The lysis pattern of the targets loaded with the different Uroplakin II homologue peptides was similar to the CTL  
25 assays using human PBL, CTL from HhD mice immunized with Uroplakin II peptides or with TCC peptide extract (Figure 5).

Although human and HhD murine CTL recognize the same UP II peptides, it was not clear whether HhD murine CTL are directed against the actual T cell epitopes or against possible differences between humanoid and rodent UP II. We  
30 evaluated also the crossreactivity of CTL from mice immunized with human Uroplakin II peptides against 4 homologue murine Uroplakin II peptides. The murine UP II sequence was screened for HLA-A2 binding peptides and 4 peptide homologues to human peptides 1, 3, 4 and 6 were synthesized. Murine peptides differ in 1-3 amino acids from similar human peptides, yet most are conservative  
35 changes (Tables 1 and 3). Murine UP II peptides bind stably to RMA-S-HhD cells.

There was significant lysis of the targets loaded with each of these murine peptides. A good correlation between the LU30 results for the human and their

homologue murine peptides was found. Three human peptides induced more target lysis than their murine counterpart and for the fourth peptide the trend was opposite (Figures 4 and 5). Thus, the specificity of the HhD murine CTL, as the specificity of human PBL is directed to common epitopes on the peptides. To  
5 examine whether any autoimmune effects are observed in peptide vaccinated HhD mice, the internal organs of the HhD mice immunized with TCC peptide extract or with Uroplakin II homologue peptides were formalin fixed and representative sections from each organ were stained with hematoxylin and eosin. Two non-immunized HhD mice served as control. There were no pathological finding  
10 such as tissue necrosis or inflammatory infiltrate in any of the organ examined. The urinary bladder was carefully examined using a larger number of sections. The bladder wall including the mucosa was normal in the immunized and in the control mice.

The immunogenicity of additional Uroplakin-derived peptides was  
15 evaluated in the HhD mouse strain, in which the murine MHC class I genes are not expressed (Db<sup>-/-</sup>,  $\beta$ 2-microglobulin<sup>-/-</sup> double knock-out), and which is transgenic for an HLA-A2 and human  $\beta$ 2-microglobulin monochain. Seven HLA-A2 binding peptides derived from the sequence of Uroplakin Ib, 2 from Uroplakin II, and 6 from Uroplakin III (see Table I) were tested for their ability to  
20 evoke specific CTL responses in these mice. After three weekly immunizations, splenic lymphocytes were restimulated *in vitro* with cognate peptide and then incubated with radiolabeled peptide-loaded target cells. Figures 23a-e show that several of the tested peptides (peptides B1, B2, B5, B6, 3.2, 3.3, and 8) could elicit lymphocytes which specifically lysed target cells. Importantly, in all assays there  
25 was no significant lysis of unloaded target cells (non) or of cells loaded with a non-specific HLA-A2 binding peptide from the melanoma antigen tyrosinase (tyr).

Table 3a below shows the sequences of the peptides tested in single letter amino acid code, their starting position in the intact protein.

**TABLE 3a**  
***Predicted human Uroplakin Ib, II and III peptides that bind to HLA-A2***

Peptide	Start Position	Sequence
Uroplakin Ib/B1	239	AILCWTFWV
Uroplakin Ib/B2	92	FILMFIVYA
Uroplakin Ib/B3	29	LTAECIFFV
Uroplakin Ib/B4	154	MLQDNCCGV
Uroplakin Ib/B5	240	ILCWTFWVL
Uroplakin Ib/B6	86	KILLAYFIL
Uroplakin Ib/B7	64	FVGICLFCL
Uroplakin II/8	161	VLLSVAMFL
Uroplakin II/9	162	LLSVAMFLL
Uroplakin III/3.1	214	ILGSLPFFL
Uroplakin III/3.2	128	ILNAYLVRV
Uroplakin III/3.3	221	FLLVGFGA
Uroplakin III/3.4	20	NLQPQLASV
Uroplakin III/3.5	47	CMFDSKEAL
Uroplakin III/3.6	62	YLYVLVDSA
Tyrosinase	368	YMDGTMSQV

Table 3a shows the sequences of the peptides tested in single letter amino acid code and their starting position in the intact protein (according to NCBI accession nos. 3298345 (peptides 3.1-3.6), 3483011 (peptides 8 and 9), and 3721858 (peptides B1-B7)).

## EXAMPLE 2

**PSA, PSMA and PAP homologue peptides are immunogenic CTL epitopes in HLA-A2 transgenic H-2D<sup>b</sup>,  $\beta$ 2m double knockout mice**

Carcinoma of the prostate (CAP) is the most prevalent cancer and the second cause of death in man (53). The use of prostate specific antigen (PSA) in early detection of CAP has caused a stage shift of the disease (54). By now, 70% of CAP cases are detected when they are still organ confined. These patients may be cured by radical prostatectomy or by radiation therapy. However, the long term cancer specific survival of organ confined disease after radical prostatectomy, even in the best series, is only 70 % (55). Patients with large tumors, high grade tumors and those with seminal vesicle invasion, or positive lymph nodes are at increased risk of metastases and death due to CAP (56). However, CAP is a slow growing tumor and there is no effective adjuvant chemotherapy (57). Androgen ablation by surgical or medical means does not

increase survival, but only increases time to recurrence and palliate symptomatic bone metastases (58). There is a need for an effective adjuvant or neo-adjuvant therapy for CAP and for systemic therapy for metastatic CAP. Thus, specific immunity is being investigated as a potential adjuvant therapy. PSA and prostate specific membrane antigen (PSMA) are proteins which are expressed almost exclusively in prostate tissue, benign or malignant and are differentiation antigens. Prostate acid phosphatase (PAP) is expressed also in other tissues but its concentration is much higher in prostate tissue. In a few studies, 3 PSA homologue peptides (59-60) and 2 PSMA homologue peptides (61-62) were found to be immunogenic and to induce specific CTL by repeated *in vitro* stimulation of peripheral blood lymphocytes from CAP patients. In one of these studies (62), patients with metastatic prostate cancer were immunized with the 2 immunogenic PSMA peptides, loaded on dendritic cells. There was some partial responses expressed by a reduction in PSA levels and some objective reduction in bone metastases burden. However, the procedures used to induce CTL lines *in vitro* are cumbersome, lengthy and not reliable. Some CTL peptide epitopes which bind with high affinity to MHC class I may induce tolerance and will be not detected. Moreover, repeated *in vitro* stimulations, necessary for inducing CTL lines may select CTL which have a growth advantage in culture and not the most potent and specific CTL *in vivo*. Herein the HLA-A2 transgenic, H-2D<sup>b</sup> β2m<sup>-/-</sup> double knockout mice (HhD mice) was used for identification of novel prostate cancer specific CTL epitopes. Peptides homologue to PSA, PSMA and PAP, which are expressed mainly in benign or malignant prostate tissue, were evaluated for their capacity to induce specific CTL in HhD mice. The amino acid sequences of these proteins (NCBI accession Nos. 296671, 2897946 and 439658) were screened by a HLA binding motif program. The peptides which were predicted to bind to HLA-A2 were synthesized (Tables 4, 5, 6) and their binding affinity was tested on RMA-S-HhD cells (TAP deficient cells, capable of presenting only exogenous peptides and which were transfected with the HhD construct) by FACS (Figures 6 and 7). The peptides with high binding affinity were used for immunization of HhD mice. Five PSA homologue peptides, in addition to the peptides detected by other authors, 6 PSMA homologue peptides, including the 2 peptides detected by other authors (PSMA 5 and 6) and 4 PAP homologue peptides were found.

35

TABLE 4

***Predicted human prostate specific antigen (PSA) peptides that bind to HLA-A2***

Peptide	Sequence	Amino-acids*	SEQ ID:
PSA1	DLHVISNDV	175-183	20



PSA2	VLVHPQWVL	53-61	21
PSA3	FLRPGDDSS	110-118	22
PSA4	ALGTTCYAS	147-155	23
PSA5	KLQCVDLHV	170-178	24

- 5 The prediction program is described in Materials and Methods. The peptides are listed in descending order of predicted HLA-A2.1-peptide complex stability. Single letter amino acid codes and the position of the peptide in the protein are listed. \* Numbering is according to NCBI accession No. 296671.

10

TABLE 5

*Predicted human prostate specific membrane antigen (PSMA) peptides that bind to HLA-A2*

Peptide	Sequence	Amino-acids*	SEQ ID:
PSMA1	ELAHYDVLL	109-117	25
15 PSMA2	NLNGAGDPL	260-268	26
PSMA3	TLRVDCTPL	461-469	27
PSMA4	MMNDQLMFL	663-671	28
PSMA5	ALFDIESKV	711-719	29
PSMA6	LLHETDSAV	4-12	30

- 20 The prediction program is described in Materials and Methods. The peptides are listed in descending order of predicted HLA-A2.1-peptide complex stability. Single letter amino acid codes and the position of the peptide in the protein are listed. \* Numbering is according to NCBI accession No. 2897946.

25

TABLE 6

*Predicted human prostate acid phosphatase (PAP) peptides that bind to HLA-A2*

Peptide	Sequence	Amino-acids*	SEQ ID:
PAP1	VLAKELKFV	30-38	31
30 PAP2	ILLWQPIPV	135-143	32
PAP3	DLFGIWSKV	201-209	33
PAP4	PLERFAELV	352-360	34

- The prediction program is described in Materials and Methods. The peptides are listed in descending order of predicted HLA-A2.1-peptide complex stability.
- 35 Single letter amino acid codes and the position of the peptide in the protein are listed. \* Numbering is according to NCBI accession No. 439658.

HhD mice were immunized and boosted twice with the PSA, PSMA, or PAP peptides. Peptides were loaded individually on RMA-S-HhD cells and equal numbers of pulsed cells were mixed in the vaccine. Mice were also immunized with CAP peptide extract, or with DU145-HhD, a CAP cell line transfected with the HhD construct. Lymphocytes were obtained from the spleen of these mice and restimulated once, *in vitro* with the corresponding peptide tumor extract or cells. PSA, PSMA, PAP, CAP and normal prostate peptide extract and DU145-HhD cells were screened by the CTL obtained from these mice. The results are expressed as lytic units (LU) which represent  $10^6$ /Number of CTL effectors which induce 30 % specific target lysis. More lytic units signify a more potent CTL response.

CTL from HhD mice immunized with PAP peptides induced intense lysis of RMA-S-HhD cells (targets) loaded with PAP homologue peptides (20 to 65 LU, Figure 8). Three out of 5 PSA homologue peptides induced significant and specific lysis of targets loaded with the corresponding peptide (5.2 to 11.4 LU, Figure 9). Four out of 6 PSMA homologue peptides induced significant lysis of the targets loaded with the corresponding peptide (5 to 17 LU, Figure 10). Two out of these 4 peptides were found to be immunogenic by other authors, by *in vitro* stimulation of human peripheral blood lymphocytes. CTL obtained from each group of immunized HhD mice induced significant lysis of targets loaded with CAP peptide extract (4.5 to 22 LU). There was significantly less lysis of normal prostate peptide extract loaded targets than of the CAP peptide extract loaded targets for each immunizing peptide (Figures 8, 9 and 10). Also, there was intense lysis of DU-145-HhD cells by CTL obtained from HhD mice immunized with these cells (Figure 11), or immunized by PAP homologue peptides, or CAP peptide extract (28, 19 and 11 LU, respectively, Figure 12). There was no significant lysis of DU145-HhD cells by CTL derived from HhD mice immunized with PSA, or PSMA homologue peptides. These findings result probably from low expression of PSA and PSMA by DU145 cells.

There was good cross reactivity between CTL derived from mice immunized with CAP peptide extract and PSA, PSMA and PAP homologue peptides, which were found to be immunogenic in HhD mice. The CTL derived from mice immunized with DU145-HhD cells cross reacted with the immunogenic PAP homologue peptides (11 to 26 LU) CAP peptide extract (12 LU), and less with normal prostate peptide extract (3 LU). Overall, there was no lysis of targets loaded with nonspecific peptides such as breast cancer peptide extract, or HLA-A2 binding tyrosinase homologue peptide. Also, there was no lysis of RMA-S-HhD cells without peptides and there was no lysis of non transfected DU 145 cells

(HLA-A2 negative). The internal organs of the HhD mice immunized with PSA, PSMA, PAP homologue peptides, or with CAP peptide extract, or with DU145-HhD cells, were formalin fixed and representative slices from each organ were stained with hematoxyllin and eosin. Two non-immunized HhD mice served  
5 as control. There was no pathological finding such as tissue necrosis or inflammatory infiltrate in any of the organ examined.

**EXAMPLE 3****Breast specific CTL induction by BA-46 (Lactadherin) peptides and by MUC1 peptides in HhD mice**

5 Breast cancer is the second leading cause of cancer death among women in the Western World and the leading cause of death among women at the age of 30 to 70. Breast cancer afflicts 200,000 women per annum in the USA today. The highest mortality is restricted to patients whose regional lymph nodes are involved. Early detection, followed by surgery provides good prognosis. In  
10 patients with occult lymph node metastasis, adjuvant chemotherapy or hormonal therapy for breast cancer have been proven to be effective, yet a large fraction of patients will succumb to metastasis. (63).

As pointed out above, TAA vaccines may constitute an additional treatment modality for residual disease. A number of tumor associated antigens have been  
15 described for breast carcinomas. The MUC-1 Mucin, a high molecular weight glycoprotein is highly expressed on breast carcinomas. Specific, MHC-unrestricted recognition of MUC-1 by human cytotoxic T cells were demonstrated (64). More recently peptides from MUC-1 were shown also to induce MHC class I and MHC class II restricted responses (65, 66). HER2/neu  
20 derived peptides were shown to be recognized by CTL from breast carcinoma patients (67).

BA-46 is a 46 kDa transmembrane-associated glycoprotein of the human milk fat globule membrane (HMFG), that is overexpressed in human breast carcinomas (68). The protein contains cell adhesion sequences (RGD), supports  
25 RGD based adhesion and interacts with integrin (69). It also contains an EGF-like domain, and a phospholipid binding sequence C1/C2-like domain of coagulation factor V and VIII. BA-46 is present in the circulation of breast cancer patients but not in healthy individuals (70). Moreover, anti BA-46 radio-conjugated monoclonal antibodies, have successfully targeted human breast tumors  
30 transplanted into mice (71). Herein we tested MUC1 and BA-46 derived peptides as potential TAA peptides in the HhD mouse system. BA-46 homologous peptide vaccines induce HLA-A2 restricted CTL which preferentially recognize breast tumor derived peptides.

BA-46 is overexpressed in many breast carcinomas, yet, no cellular  
35 immunity to BA-46 protein has been reported so far. We evaluated peptides complementary to the amino acid sequences of human BA-46, The sequence was screened for HLA-A2 binding motifs by a HLA binding motif program. Seven

9-mer peptides which were predicted to bind with high affinity to HLA-A2 were selected and synthesized (Table 7).

TABLE 7

5 ***Predicted human breast associated BA-46 peptides that bind to HLA-A2***

Peptide	Sequence	Amino-acids*	SEQ ID:
BA-46-1	KQGNFNAWV	271-279	35
BA-46-2	NLLRRMWVT	131-139	36
BA-46-5	NLFETPILA	356-364	37
10 BA-46-6	NLFETPVEA	194-202	38
BA-46-7	GLQHWVPEL	97-105	39
BA-46-8	VQFVASYSKV	313-321	40
BA-46-9	RLLAALCGA	5-13	41

The prediction program is described in Materials and Methods. The peptides are  
 15 listed in descending order of predicted HLA-A2.1-peptide complex stability. Single letter amino acid codes and the position of the first amino acid of the peptide in the protein sequence as well as the calculated binding score are listed. \* Numbering is according to NCBI accession No. 1589428.

20 All peptides bound well to the HhD molecules expressed on RMA-S transfectant (Figure 13). HhD mice were vaccinated three times at weekly interval using  $2 \times 10^6$  RMA-S-HhD/B7.1 with either tumor extract prepared from 5 samples of breast carcinoma by acid extraction and separation of molecules < 3 Kd, or with a pool of BA-46 peptides. Ten days after the last immunization, HhD  
 25 spleen-derived antigen presenting cells (APC) were pulsed for 3 hour at 37°C with either breast tumor extract or a BA-46 peptide pool, followed by incubation with the rest of the splenocytes for 4 more days. BA-46 peptides, breast extracted peptides and normal breast extracted peptides were loaded on labeled RMA-S-HhD cells and were screened by cytotoxic T lymphocytes (CTL) obtained  
 30 from the HhD mice as described before (62).

Anti BA-46 peptide CTL activity showed variability in immunogenicity at a range between 20 % to 60 %. Lysis of 60 % was obtained against BA-46-7, 40 % against BA-46-6, BA-46-9 and 20 % against the rest of the peptides (Figure 14). CTL from HhD mice immunized with breast tumor extract peptide induced  
 35 10% to 30 % specific lysis of BA-46 peptide loaded RMA-S-HhD cells, 31 % lysis of tumor extract loaded targets and only 12 % and 14 % lysis of normal breast extracted peptides and colon extracted peptides loaded RMA-S-HhD cells, respectively. No lysis of the melanoma associated synthetic tyrosinase peptide

was obtained (Figure 15). The ability of BA-46 peptides to induce a breast associated CTL reaction that is HLA-A2.1 restricted was further examined. CTL against individual BA-46 peptides showed 30-50 % higher activity against breast tumor extract versus normal breast loaded target cells, supporting the fact that a preferential activity is obtained against breast tumor TAAs. These effectors also lysed preferentially a breast carcinoma line-HhD transfectant relative to parental cells, stressing the HLA-A2 restriction of the reaction (Figures 16 and 17).

#### EXAMPLE 4

##### 10      **Characterization of novel breast carcinoma MUC1 derived peptides as CTL epitopes**

The polymorphic epithelial Mucin, encoded by the MUC-1 gene is a high molecular weight transmembranal glycoprotein overexpressed in a broad range of tumors, such as breast, pancreas, ovary, thyroid and myeloma (72) cancer. It was found that the growth rate of primary breast tumors induced by the polyoma middle T antigen is significantly slower in MUC-1 null mice, suggesting that MUC-1 might play a role in the progression of mammary carcinoma (73). Furthermore, high level of MUC-1 expression by human breast cancer cells was found to be directly correlated with the presence of axially lymph node metastasis (74). A major feature of the MUC-1 molecule is the presence of an highly immunogenic extracellular tandem repeat array (TRA) heavily O-glycosidic-linked serine and threonine residues (75). Altered carbohydrate structure of MUC-1 in breast cancer cells, is probably responsible for the exposure of core epitopes within MUC-1, specifically recognized by monoclonal antibodies, as well as non MHC-restricted cytotoxic T lymphocytes (76). HLA-A11 (77) and more recently for HLA-A2.1 (78) restricted responses to the extracellular TRA have also been reported. However it was suggested that Mucin can actively suppress cell-mediated response against glycosylated TRA (79), or induced direct T cell apoptosis (80). More recently Agrawal et al showed that synthetic peptides derived from MUC-1 TRA cause suppression of human T-cell proliferative responses (81). This data points out the ambiguous role of MUC-1 TRA in T cell activation.

A series of peptides derived from non-TRA domains of the MUC-1 as potential CTL epitopes. The MUC-1 sequence was screened for potential HLA-A2.1 binding peptides (Table 8) and the eight peptides were synthesized.

TABLE 8

*Predicted human breast associated MUC-1 peptides that bind to HLA-A2*

Peptide	Sequence	Amino-acids*	SEQ ID:
MUC1-/C6	LLLLTVLTV	31-40	42
5 MUC1-/D6	LLLTVLTVV	32-41	43
MUC1-/F6	FLSFHISNL	323-331	44
MUC1-/A5	LLVLVCVLV	442-451	45
MUC1-/F4	ALLVLVCVL	441-450	46
MUC1-/B5	SLSYTNPAV	519-528	47
10 MUC1-/A7	NLTISDVSV	412-421	48
MUC1-/E6	ALASTAPPV	226-234	49

The prediction program is described in Materials and Methods. The peptides are listed in descending order of predicted HLA-A2.1-peptide complex stability. Single letter amino acid codes and the position of the first amino acid of the peptide in the protein sequence as well as the calculated binding score are listed. \*  
 15 Numbering is according to NCBI accession No. 182253.

These 9 residue peptides are derived from the signal peptide, cytoplasmic and extracellular domains of the MUC-1 protein. Among these peptides only the  
 20 MUC-1/B5 peptide has an identical sequence to the murine homologue. HLA-A2.1 binding of MUC-1- derived peptides were evaluated by FACS analysis. The selected peptides were loaded on the murine TAP-deficient RMA-S-HhD transfectants and MHC stabilization was monitored (Figure 18). Although all peptides bound efficiently at the 1-100  $\mu$ M range, 3 peptides  
 25 MUC-1/D6, MUC-1/E6 and MUC-1/A7 exhibited higher binding affinity. In addition, similar binding affinities of these peptides were obtained upon loading on human TAP deficient T2 cells, expressing endogenous HLA-A2.1 molecules (data not shown).

Synthetic peptides corresponding to the MUC-1 TRA epitopes were shown  
 30 to induce CTL reaction in patients (77) as well as in HLA-A2.1K<sup>b</sup> transgenic mice (78). Hence we primarily examined the lysis pattern of each of the particular peptides listed in Table 8 following an immunization with a pool of the MUC-1-derived synthetic peptides (Figure 19). CTL results showed significant lysis of RMA-S-HhD target cells loaded either with the MUC-1/D6 peptide (38  
 35 %) or with the MUC-1/A7 peptide (30 %). Lower lysis rate of 15 % with the MUC-1/E6 and with the MUC-1/F6 peptides was demonstrated. The rest of the peptides showed only background lysis properties. Hence MUC-1/D6, MUC-1/A7 and MUC-1/E6 harbor immunogenic properties.

To determine the processing and presentation of MUC-1-derived peptides by breast carcinoma tumors, we selected the MDA-MB-157 cell line which is characterized by high MUC-1 expression and low class I expression (data not shown). Both the parental tumor cell line and its HhD transfectant (MDA-MB-157-HhD), were used as targets in CTL assays (Figure 20). Mice were immunized with either RMA-S-HhD-B7.1 cells loaded with MUC-1 selected peptides MUC-1/D6, MUC-1/A7 and MUC-1/E6, or with peptide extract derived from fresh patients' tumors. Preferential lysis of the MDA-MB-157-HhD cell line by anti MUC-1-derived peptides activated lymphocytes suggested both breast associated as well as MHC-restricted lysis. Moreover, this data strongly support the processing and presentation of non TRA associated MUC-1-derive peptides in MDA-MB-157-HhD breast carcinomas cells. Further analysis showed inhibition of lysis by anti HLA monoclonal antibody w6/32 (data not shown). In addition specific lysis of MDA-MB-157-HhD cells by CTL directed against fresh tumor extracted peptides emphasized an overlap in the peptide repertoire between the breast tumor cell line and fresh breast tumor extract.

To prove the existence of MUC-1/D6, MUC-1/A7 and MUC-1/E6 peptides in patients' derived breast tumor peptide extracts, a CTL experiment was performed by utilizing CTL against breast tumor peptide extracts as effectors against target cells presenting MUC-1 peptides or a control of normal breast tissue (Figure 21). All 3 MUC-1-derived peptides, but not the HLA-A2.1 melanoma associated peptide tyrosinase, could be recognized and lysed by anti-tumor extract CTL. RMA-S-HhD cells loaded with normal breast extract gave in different experiment between 40-50 % of the lysis induced against tumor extract loaded targets. This result is expected since a part of the tumor extract peptide repertoire consists of normal peptides.

A crucial parameter for selection of TAA peptides based vaccines are their expression frequency by tumors in comparison with normal tissues. Since MUC-1 protein is known to be overexpressed in tumors, with no tumor specific mutations, it is of obvious interest to examine the abundance of MUC-1 peptides in patients' derived normal breast tissue extract in comparison to tumor extract. CTL generated against MUC-1 peptides MUC-1/D6, MUC-1/A7 and MUC-1/E6, showed a 1.8-9.0 fold higher reactivity to tumor extract versus normal tissue extract (Figure 22). The same difference in preferential tumor versus normal tissue recognition by CTL could be detected upon vaccination with breast tumor extract peptides, supporting the window of specificity between normal and tumor tissues. These results suggest that MUC-1/D6, MUC-1/A7 and MUC-1/E6 are potential tumor associated antigen peptides.



**EXAMPLE 5*****Induction of CTL response by Teratocarcinoma-derived growth factor (CRIPTO-1) derived peptides.***

The CRIPTO-1 gene (also known as Teratocarcinoma-derived growth factor) was first identified and cloned from an undifferentiated human embryonal carcinoma cell line (83). It encodes a 188 amino acid glycoprotein with a region of structural homology to members of the epidermal growth factor family, but lacks a hydrophobic signal peptide and transmembrane domain. The receptor for CRIPTO-1 has not been identified, yet it has been shown to function as a growth factor. The gene can act as a dominantly transforming oncogene *in vitro*. A number of studies have shown elevated expression of CRIPTO-1 mRNA and protein in several human cancers, including human primary breast, gastric, colon, pancreatic, and bladder (84-88). It is therefore an attractive candidate tumor associated antigen for specific, active immunotherapy. Peptides have been synthesized with HLA-A2.01 binding potential based on the published sequences of CRIPTO-1, and these peptides have been used for immunization of HhD transgenic mice (see Table 9). Figures 24a-c show that some of the tested peptides (C1, C8, C10, and C12) can elicit lymphocyte response which specifically lyses CRIPTO-1 peptide-loaded target cells.

**TABLE 9*****Predicted human Cripto-1 derived peptides that bind to HLA-A2***

Peptide	Start Position	Sequence
Cripto-1/C1	5	KMARFSYSV
Cripto-1/C2	151	GLVMDEHLV
Cripto-1/C3	145	FLPGCDGLV
Cripto-1/C4	89	CMLGSFCAC
Cripto-1/C5	43	YLAFRDDSI
Cripto-1/C6	123	WLPKKCSLC
Cripto-1/C7	83	CLNGGTCML
Cripto-1/C8	176	MLVGICLSI
Cripto-1/C9	23	FELGLVAGL
Cripto-1/C10	5	KMVRFSYSV
Cripto-1/C11	83	CLNEGTCML
Cripto-1/C12	176	MLAGICLSI

Table 9 shows the sequences of the peptides tested in single letter amino acid code and their starting position in the intact protein (according to NCBI accession nos. 117473 (C1-C9) and 321120 (C10-C12)).

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended

to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

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## WHAT IS CLAIMED IS

1. A peptide derived from a protein selected from the group consisting of Uroplakin (UP), Prostate specific antigen (PSA), Prostate specific membrane antigen (PSMA), Prostate acid phosphatase (PAP), Lactadherin (BA-46), Mucin (MUC1) and Teratocarcinoma-derived growth factor (CRIPTO-1), the peptide comprising 8 to 10 amino acid residues, of which a second residue from an amino terminal of the peptide and an end residue at a carboxy terminal of the peptide are hydrophobic or hydrophilic natural or non-natural amino acid residues.
2. The peptide of claim 1, wherein the peptide is derived from Uroplakin.
3. The peptide of claim 2, wherein said Uroplakin is selected from the group consisting of Uroplakin II, Uroplakin Ia, Uroplakin III and Uroplakin Ib.
4. The peptide of claim 3, wherein the peptide has a sequence selected from the group consisting of SEQ ID NOs:1-19 and 50-64.
5. The peptide of claim 1, wherein the peptide is derived from said Prostate specific antigen (PSA).
6. The peptide of claim 5, wherein the peptide has a sequence selected from the group consisting of SEQ ID NOs:20-24.
7. The peptide of claim 1, wherein the peptide is derived from said Prostate specific membrane antigen (PSMA).
8. The peptide of claim 7, wherein the peptide has a sequence selected from the group consisting of SEQ ID NOs:25-30.
9. The peptide of claim 1, wherein the peptide is derived from said Prostate acid phosphatase (PAP).
10. The peptide of claim 9, wherein the peptide has a sequence selected from the group consisting of SEQ ID NOs:31-34.

11. The peptide of claim 1, wherein the peptide is derived from said Mucin.
12. The peptide of claim 11, wherein the peptide is derived from a non-tandem repeat array of said Mucin.
13. The peptide of claim 11, wherein the peptide is derived from a region selected from the group consisting of a signal peptide, a cytoplasmic domain and an extracellular domain of said Mucin.
14. The peptide of claim 13, wherein the peptide is derived from a non tandem repeat array of said Mucin.
15. The peptide of claim 11, wherein the peptide has a sequence selected from the group consisting of SEQ ID NOs:42-49.
16. The peptide of claim 1, wherein the peptide is derived from said Lactadherin (BA-46).
17. The peptide of claim 16, wherein the peptide has a sequence selected from the group consisting of SEQ ID NOs:35-41.
18. The peptide of claim 1, wherein the peptide is derived from said Teratocarcinoma-derived growth factor (CRIPTO-1).
19. The peptide of claim 18, wherein the peptide has the sequence selected from the group consisting of SEQ ID Nos. 66 to 77.
20. The peptide of claim 1, wherein said Uroplakin (UP), Prostate specific antigen (PSA), Prostate specific membrane antigen (PSMA), Prostate acid phosphatase (PAP), Lactadherin (BA-46), Mucin (MUC1) and Teratocarcinoma-derived growth factor (CRIPTO-1) are each independently of mammalian origin.
21. The peptide of claim 20, wherein said mammal is selected from the group consisting of a humanoid and a rodent.

22. The peptide of claim 1 having a sequence selected from the group consisting of SEQ ID NOs:1-64 and 66 to 77.
23. The peptide of claim 1, wherein said peptide includes at least one non-natural modification.
24. The peptide of claim 23, wherein said non-natural modification renders peptides more immunogenic or more stable.
25. The peptide of claim 23 or 24, wherein said at least one modification is selected from the group consisting of peptoid modification, semipeptoid modification, cyclic peptide modification, N terminus modification, C terminus modification, peptide bond modification, backbone modification and residue modification.
26. A pharmaceutical composition comprising, as an active ingredient, at least one peptide as set forth in any of claims 1-25 and a pharmaceutically acceptable carrier.
27. The pharmaceutical composition of claim 26, wherein said carrier is selected from the group consisting of a proteinaceous carrier to which said at least one tumor associated antigen peptide is linked, an adjuvant, a protein or a recombinant protein and an antigen presenting cell.
28. The pharmaceutical composition of claim 26, wherein the composition is effective in prevention or cure of cancer or cancer metastases.
29. The pharmaceutical composition of claim 28, wherein said cancer is selected from the group consisting of breast, bladder, prostate, pancreas, ovary, thyroid, colon, stomach and head and neck cancer.
30. The pharmaceutical composition of claim 28, wherein said cancer is a carcinoma.
31. The pharmaceutical composition of claim 26, wherein the composition is a vaccine.

32. A vaccine composition comprising, as an active ingredient, at least one peptide as set forth in any of claims 1-25 and a suitable carrier.

33. The vaccine composition of claim 32, wherein said carrier is selected from the group consisting of a proteinaceous carrier to which said at least one tumor associated antigen peptide is linked, an adjuvant, a protein or a recombinant protein and an antigen presenting cell.

34. The vaccine composition of claim 32, wherein the composition is effective in prevention or cure of cancer or cancer metastases.

35. The vaccine composition of claim 34, wherein said cancer is selected from the group consisting of breast, bladder, prostate, pancreas, ovary, thyroid, colon, stomach and head and neck cancer.

36. The vaccine composition of claim 34, wherein said cancer is a carcinoma.

37. A method of prevention or cure of a cancer or of metastases thereof comprising the step of administering to a patient an effective amount of the pharmaceutical composition of any of claims 26-31.

38. A method of prevention or cure of a cancer or of metastases thereof comprising the step of vaccinating a patient with an effective amount of the vaccine composition of any of claims 32-36.

39. A polynucleotide encoding at least one peptide according to any of claims 1-25.

40. A polynucleotide encoding at least one peptide according to any of the SEQ ID Nos. 1 to 64 and 66 to 77.

41. The polynucleotide of claim 39 or 40, wherein the polynucleotide forms a part of a longer polynucleotide designed to encode a fused protein product from which said at least one peptide is cleavable by a protease.

42. A pharmaceutical composition comprising, as an active ingredient, at least one polynucleotide as set forth in any claims 39-41 and a pharmaceutically acceptable carrier.

43. A cellular vaccine composition comprising an antigen presenting cell presenting at least one peptide derived from a protein selected from the group consisting of Uroplakin (UP), Prostate specific antigen (PSA), Prostate specific membrane antigen (PSMA), Prostate acid phosphatase (PAP), Lactadherin (BA-46) Mucin (MUC1) and Teratocarcinoma-derived growth factor (CRIPTO-1), the at least one peptide comprising 8 to 10 amino acid residues, of which a second residue from an amino terminal of the peptide and a carboxy terminal residue of the peptide are hydrophobic or hydrophilic, natural or non-natural amino acid residues.

44. The cellular vaccine composition of claim 43, wherein said antigen presenting cell is selected from the group consisting of a dendritic cell, a macrophage, a B cell and a fibroblast.

45. The cellular vaccine composition of claim 43, wherein said antigen presenting cell is caused to present said at least one tumor associated antigen peptide by a method selected from the group consisting of:

- (a) genetically modifying said antigen presenting cell with at least one polynucleotide encoding said at least one tumor associated antigen peptide such that said peptide or at least one longer polypeptide including said peptide will be expressed;
- (b) loading said antigen presenting cell with at least one polynucleotide encoding said at least one tumor associated antigen peptide;
- (c) loading said antigen presenting cell with said at least one tumor associated antigen peptide; and
- (d) loading said antigen presenting cell with at least one longer polypeptide including said at least one tumor associated antigen peptide

46. The peptide of claim 1, wherein the second residue and the end residue are neutral, hydrophobic and aliphatic.

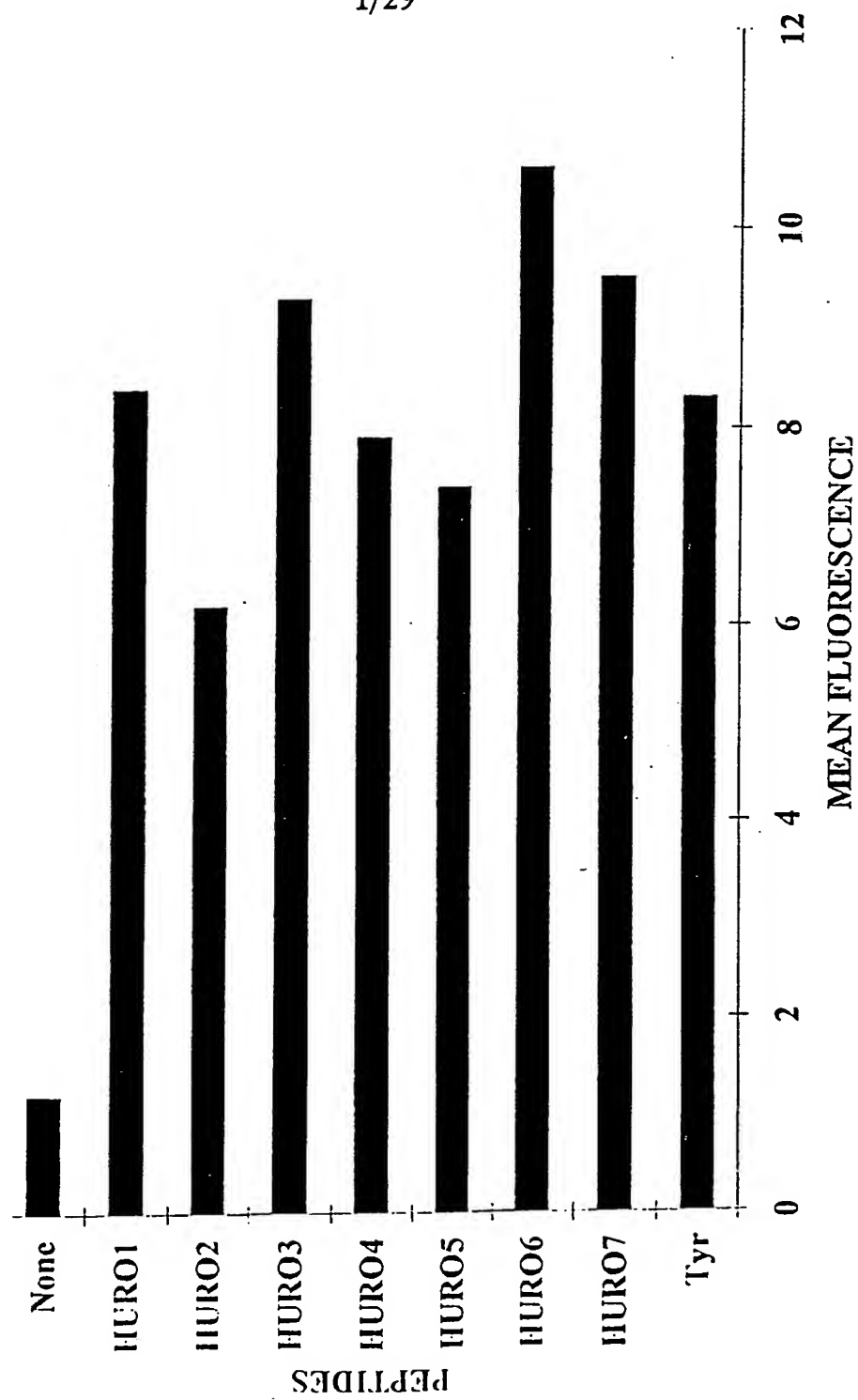
47. The pharmaceutical composition of any of claims 26 to 31 and 42 also comprising a helper peptide.



48. The pharmaceutical composition of claim 47, wherein the helper peptide has a T helper epitope.
49. The vaccine composition of any of claims 32 to 36 also comprising a helper peptide.
50. The vaccine composition of claim 49 wherein the helper peptide has a T helper epitope.
51. Use of the at least one peptide of claims 1 to 25 in the manufacture of a medicament, substantially as described in the specification.
52. The at least one peptide of claims 1 to 25 for use as a medicament.
53. Use of the at least one peptide of claims 1 to 25 in the manufacture of a medicament for the prevention or cure of a cancer or cancer metastases, substantially as described in the specification.
54. The at least one peptide of claims 1 to 25 for use as a medicament for the prevention or cure of a cancer or cancer metastases.
55. A peptide derived from a protein selected from the group consisting of Uroplakin (UP), Prostate specific antigen (PSA), Prostate specific membrane antigen (PSMA), Prostate acid phosphatase (PAP), Lactadherin (BA-46) and Mucin (MUC1) and Teratocarcinoma-derived growth factor (CRIPTO-1), the peptide comprising 8-10 amino acid residues as selected so as to promote effective binding to a MHC class I type molecule such that a CTL response is elicitable.

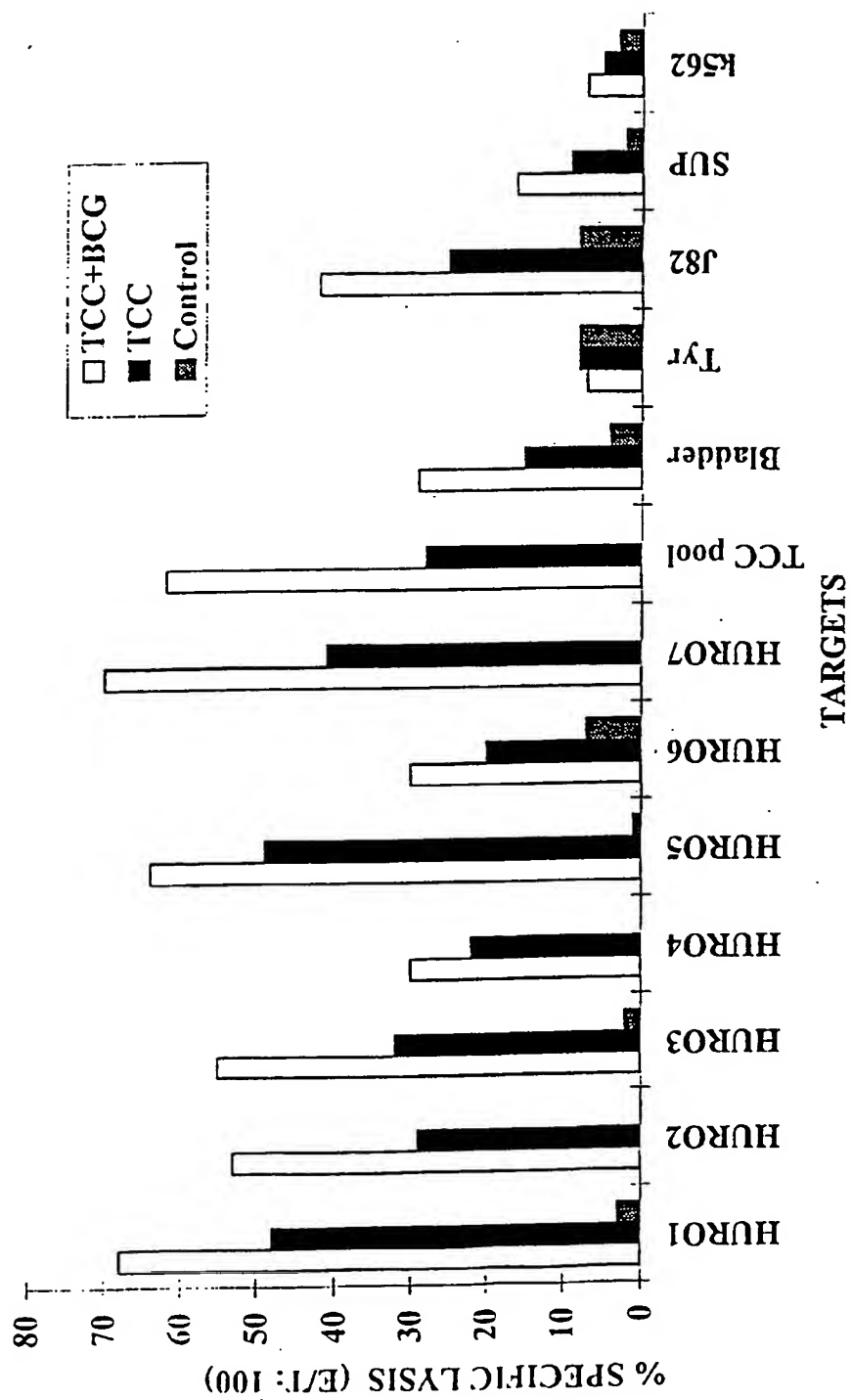
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Fig. 1



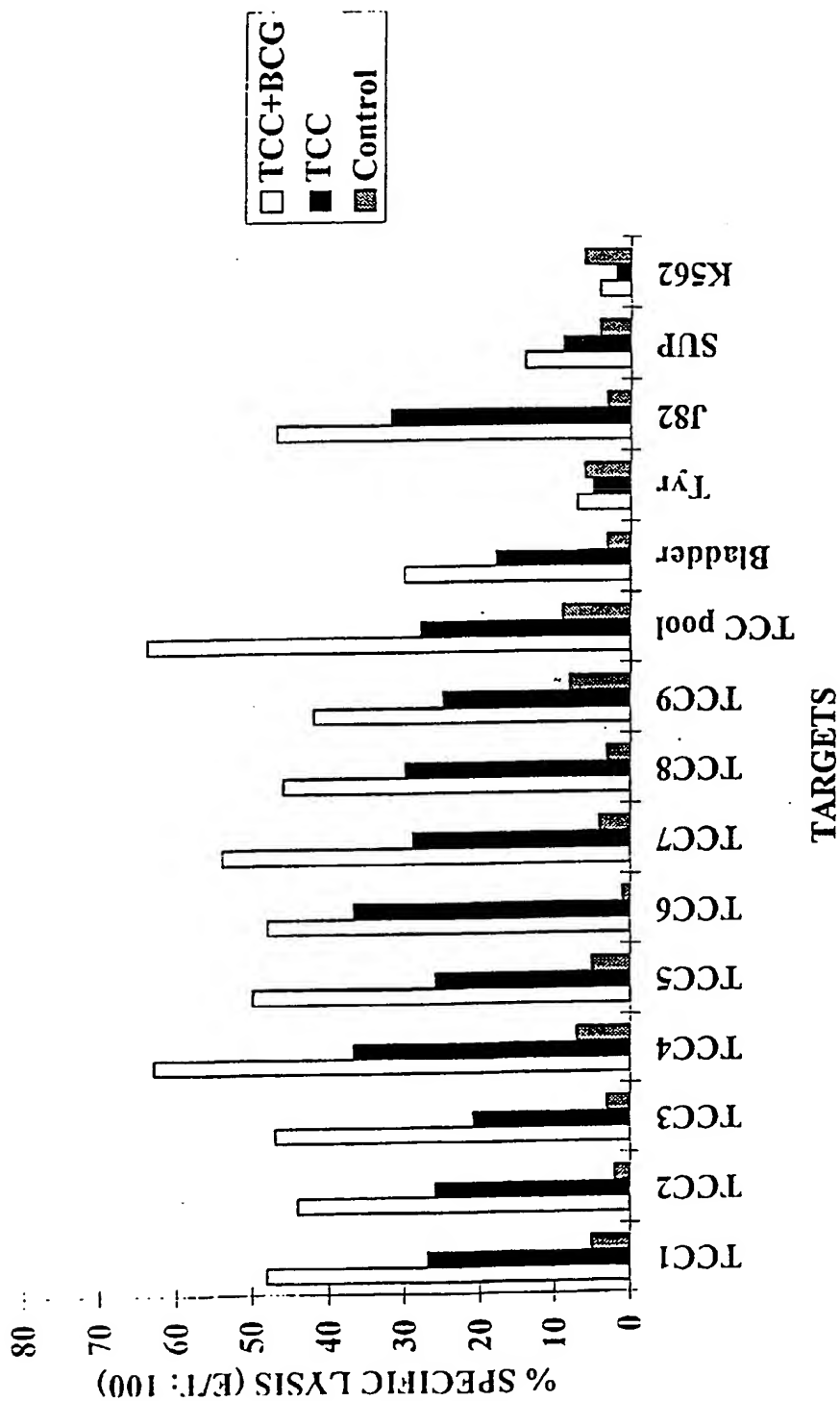
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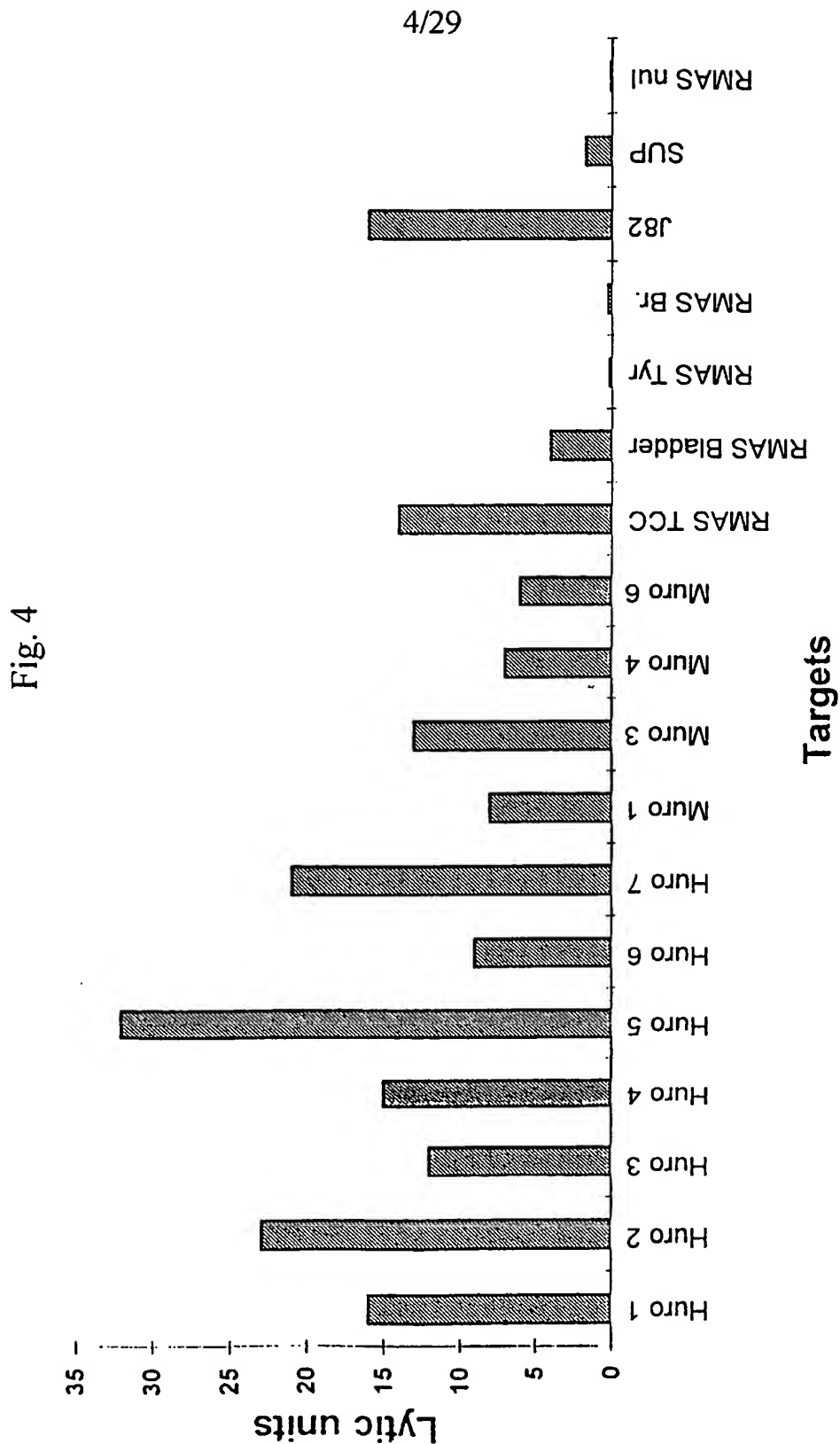
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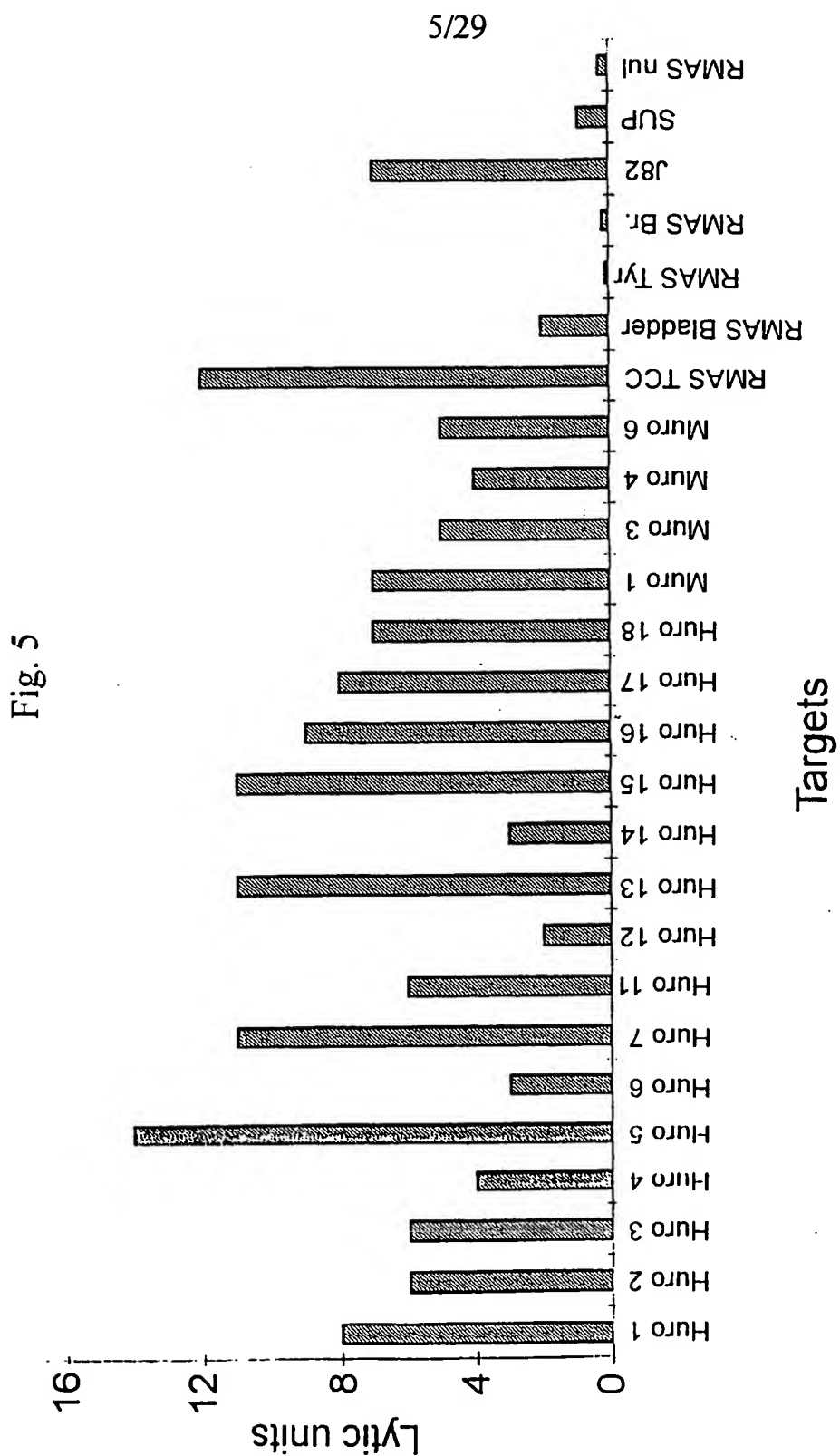


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Fig. 3

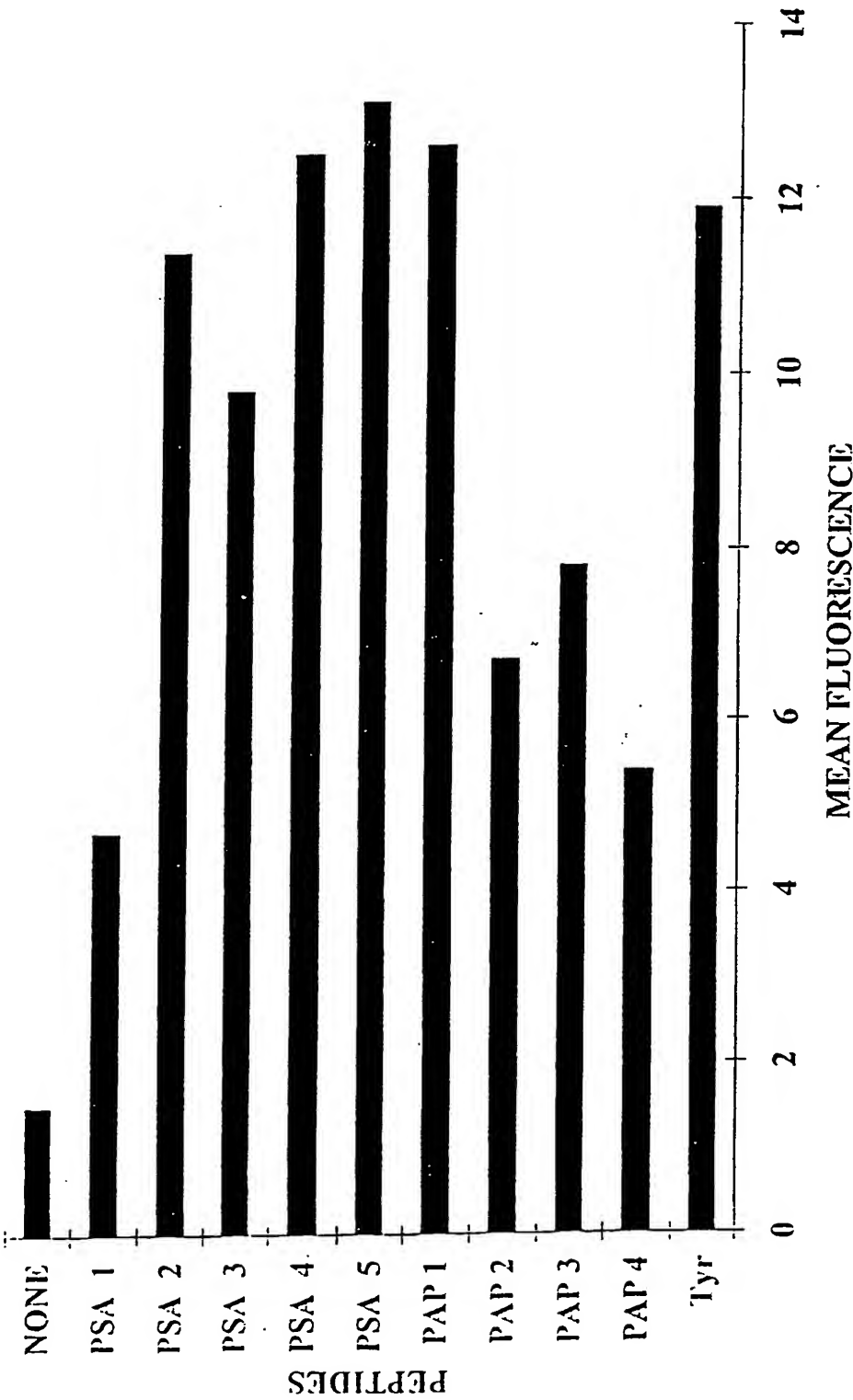






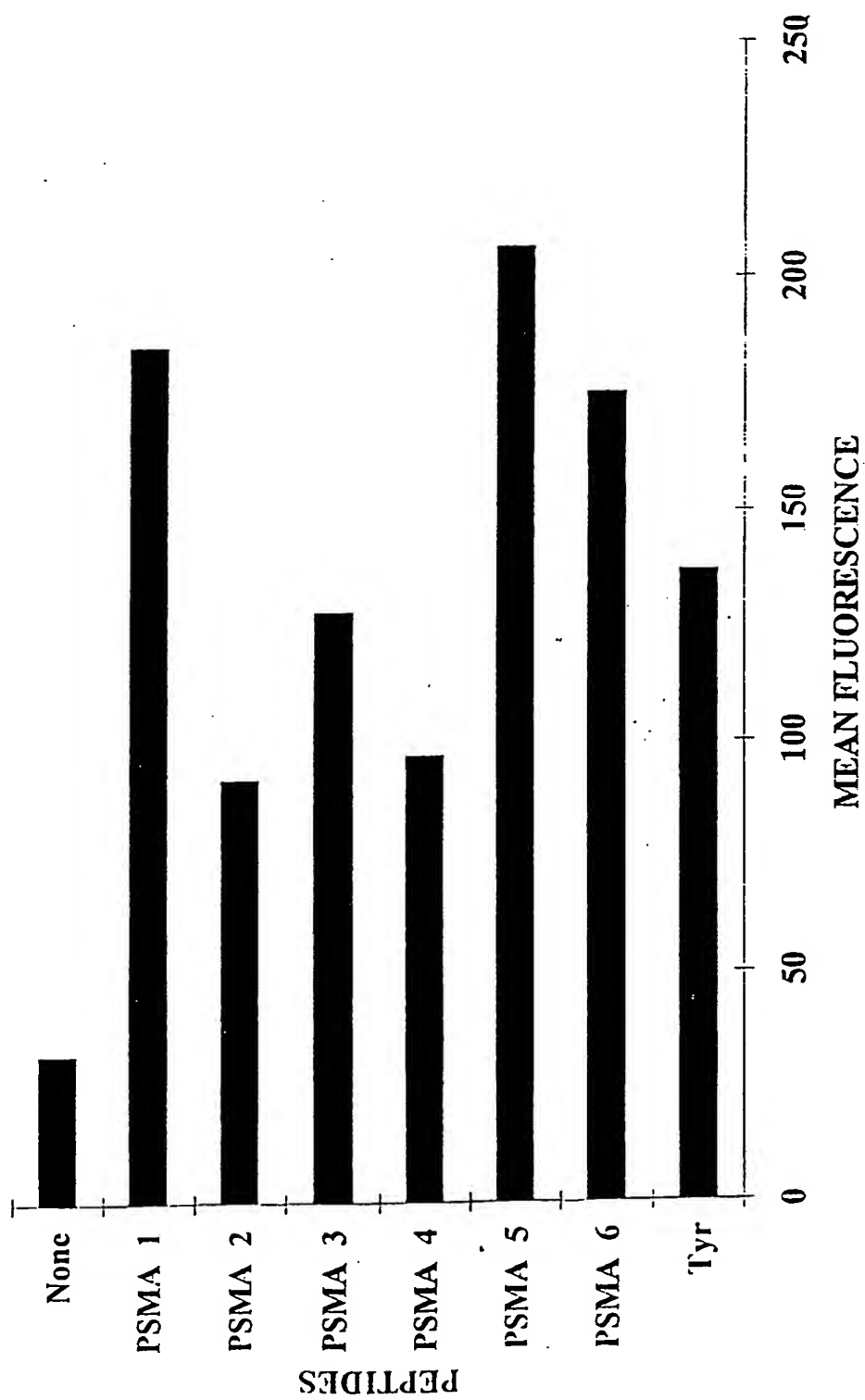
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Fig. 6



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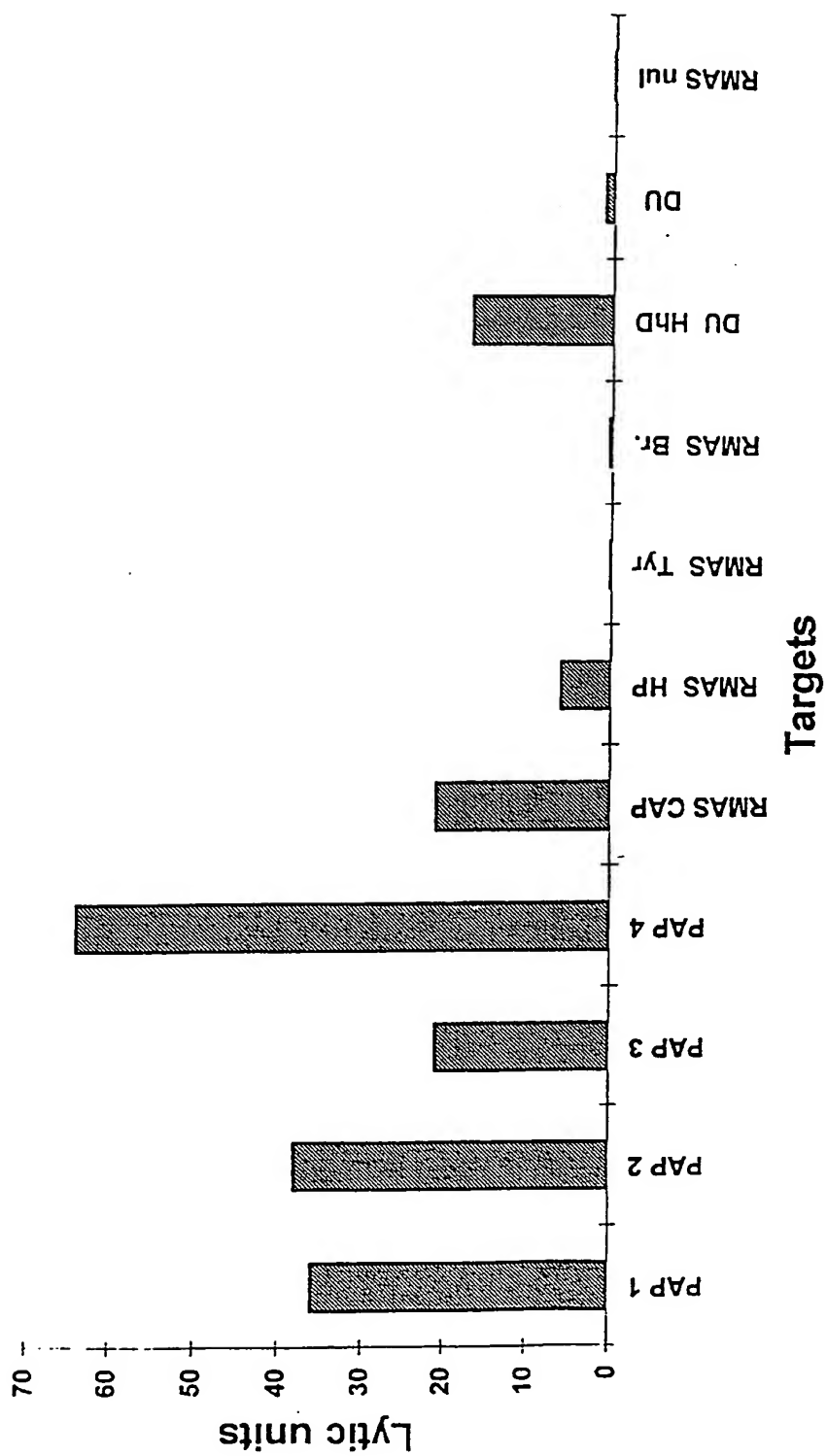
Fig. 7





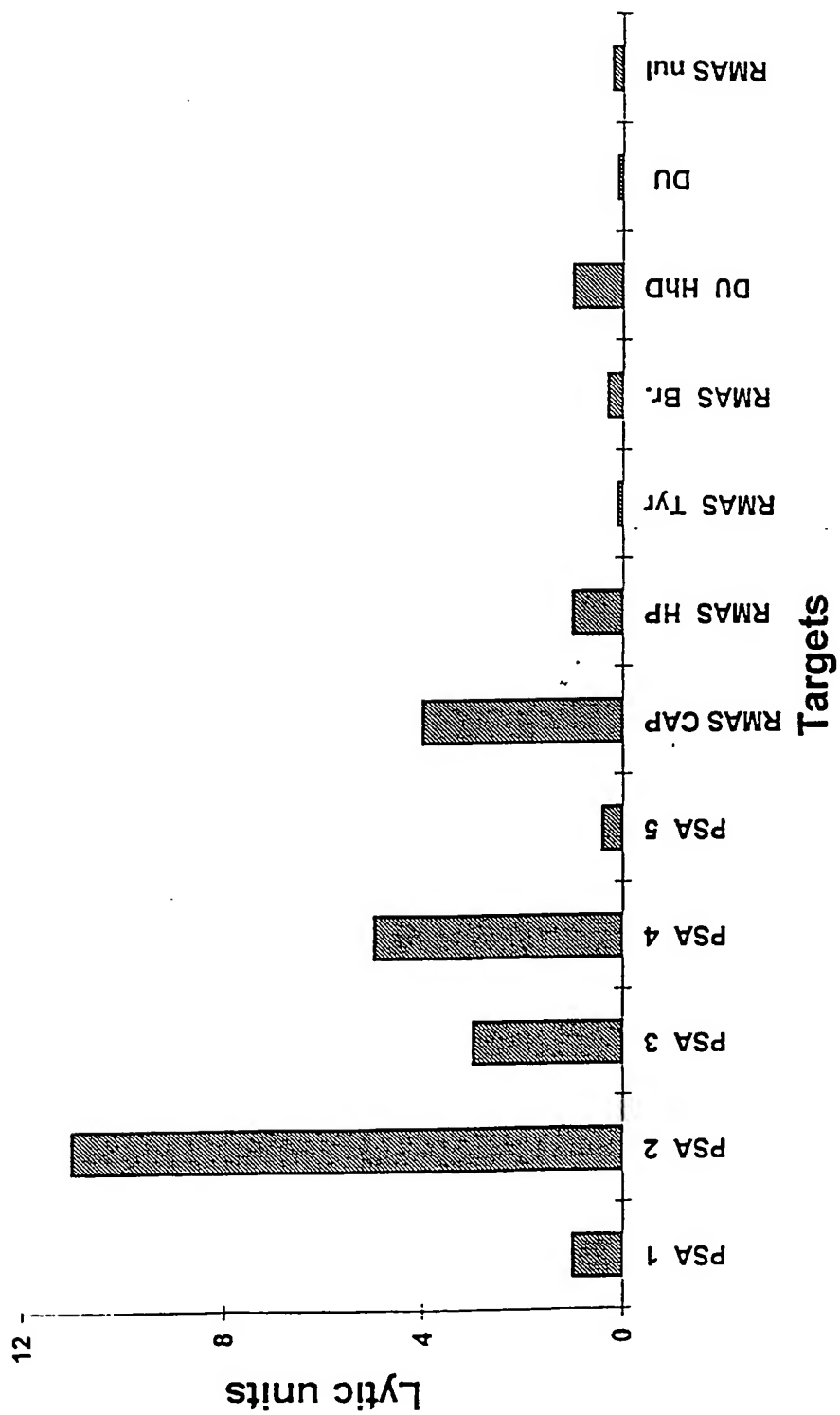
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Fig. 8



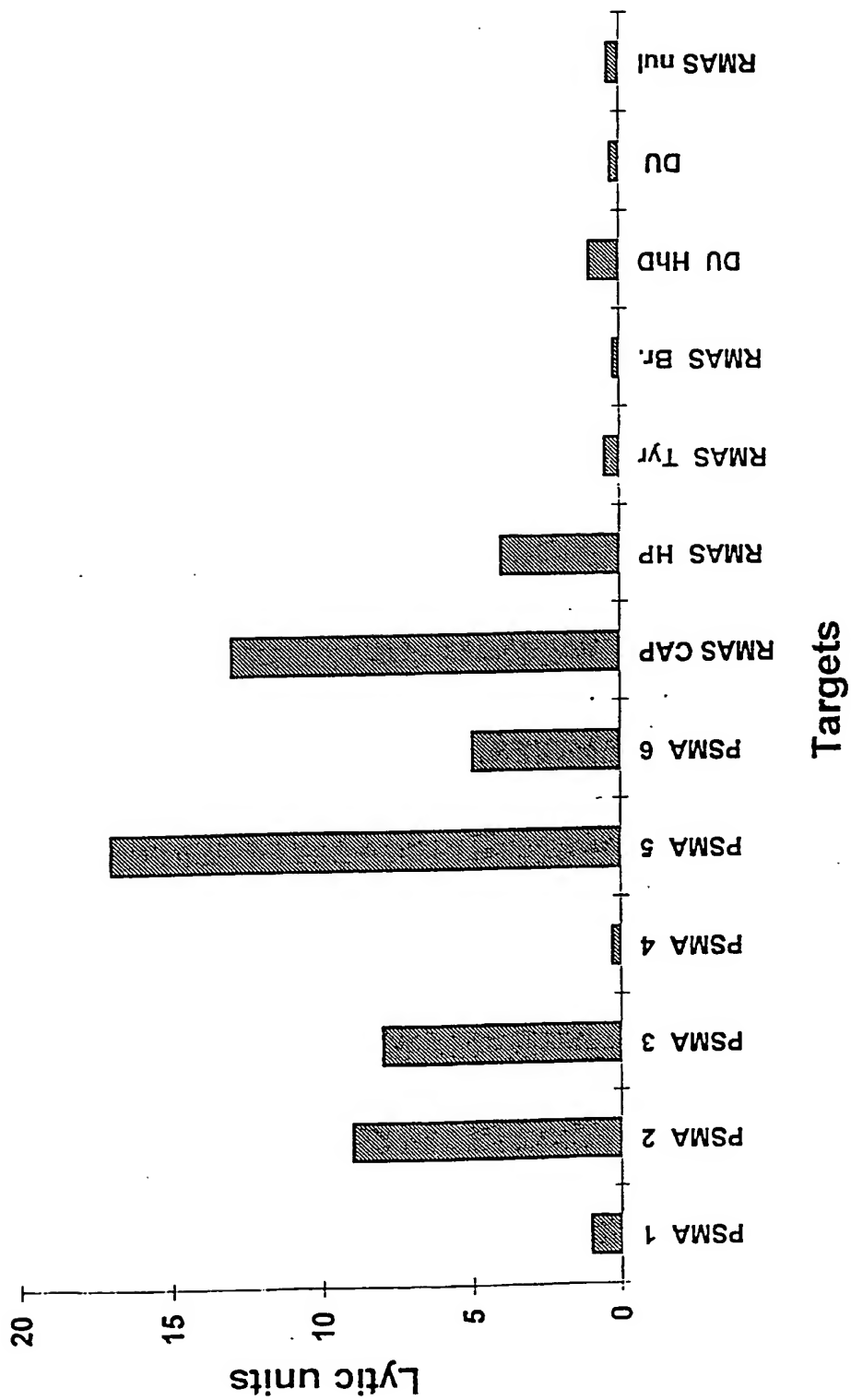
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Fig. 9



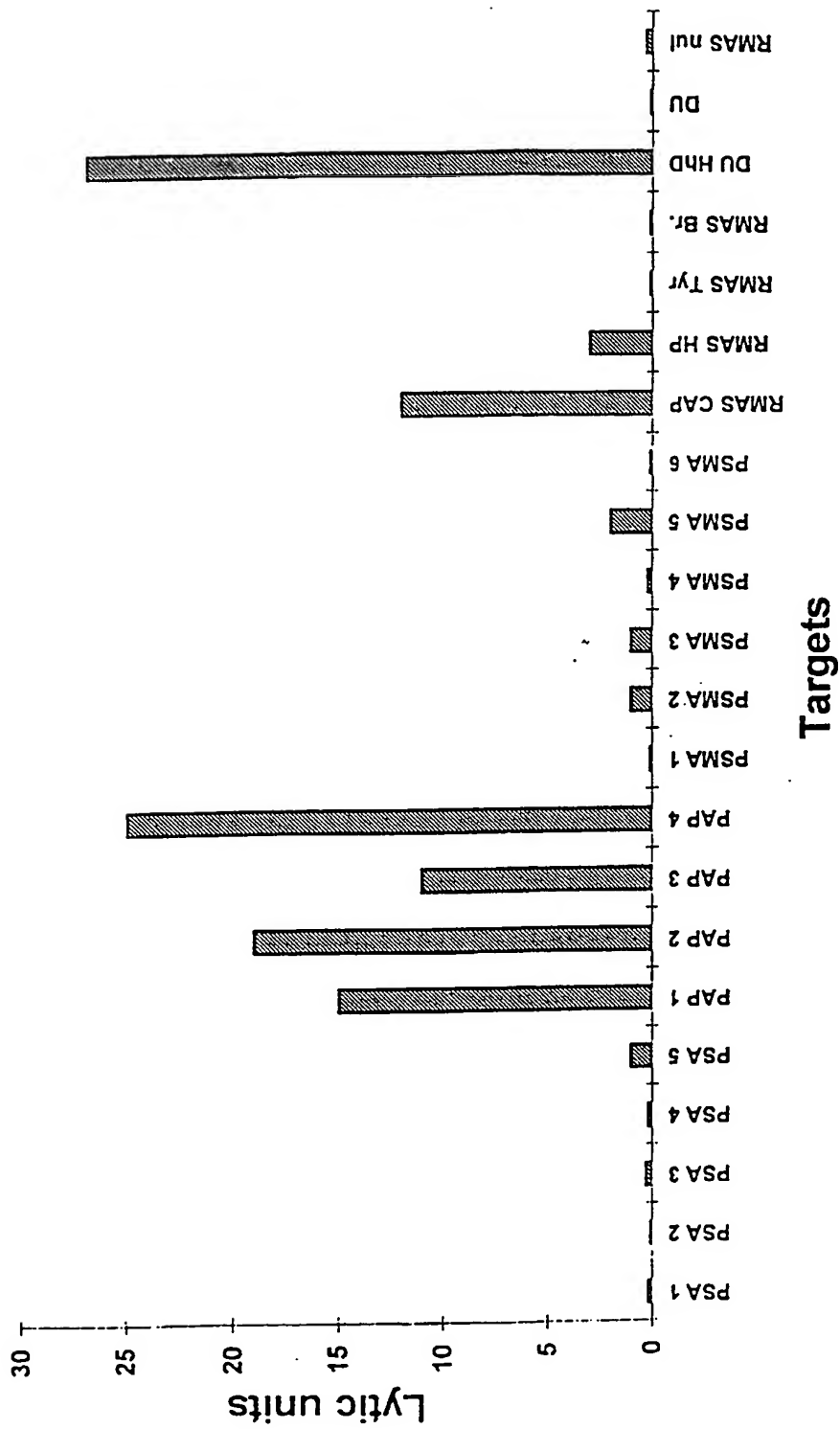
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Fig. 10



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Fig. 11



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Fig. 12

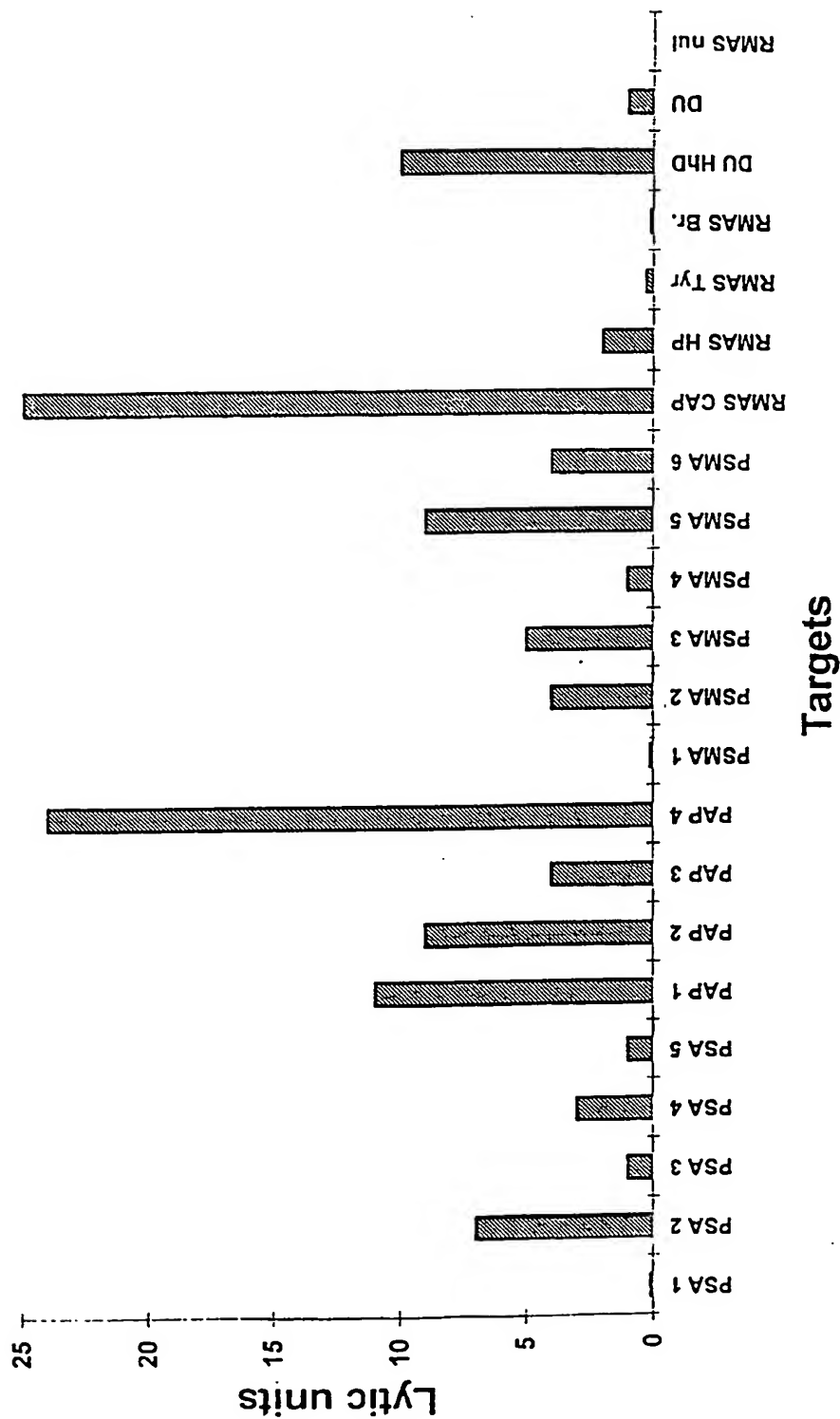
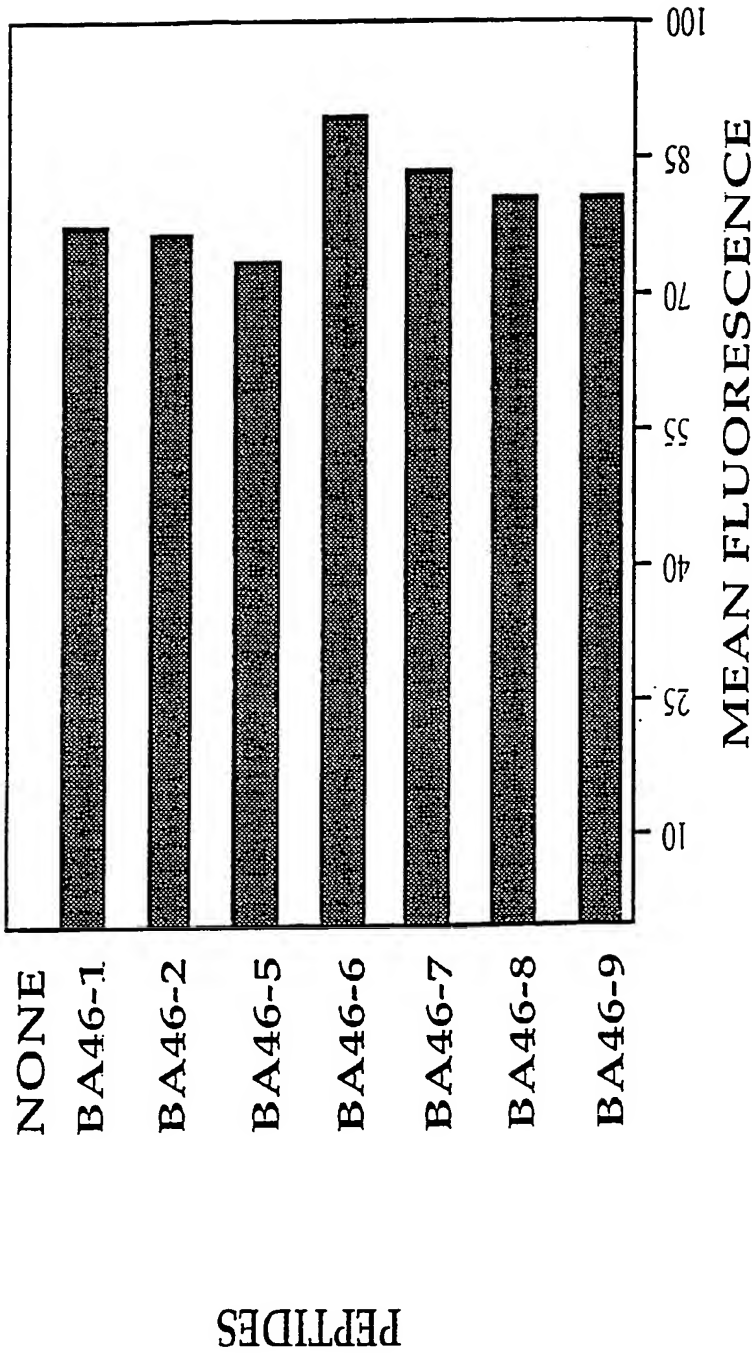
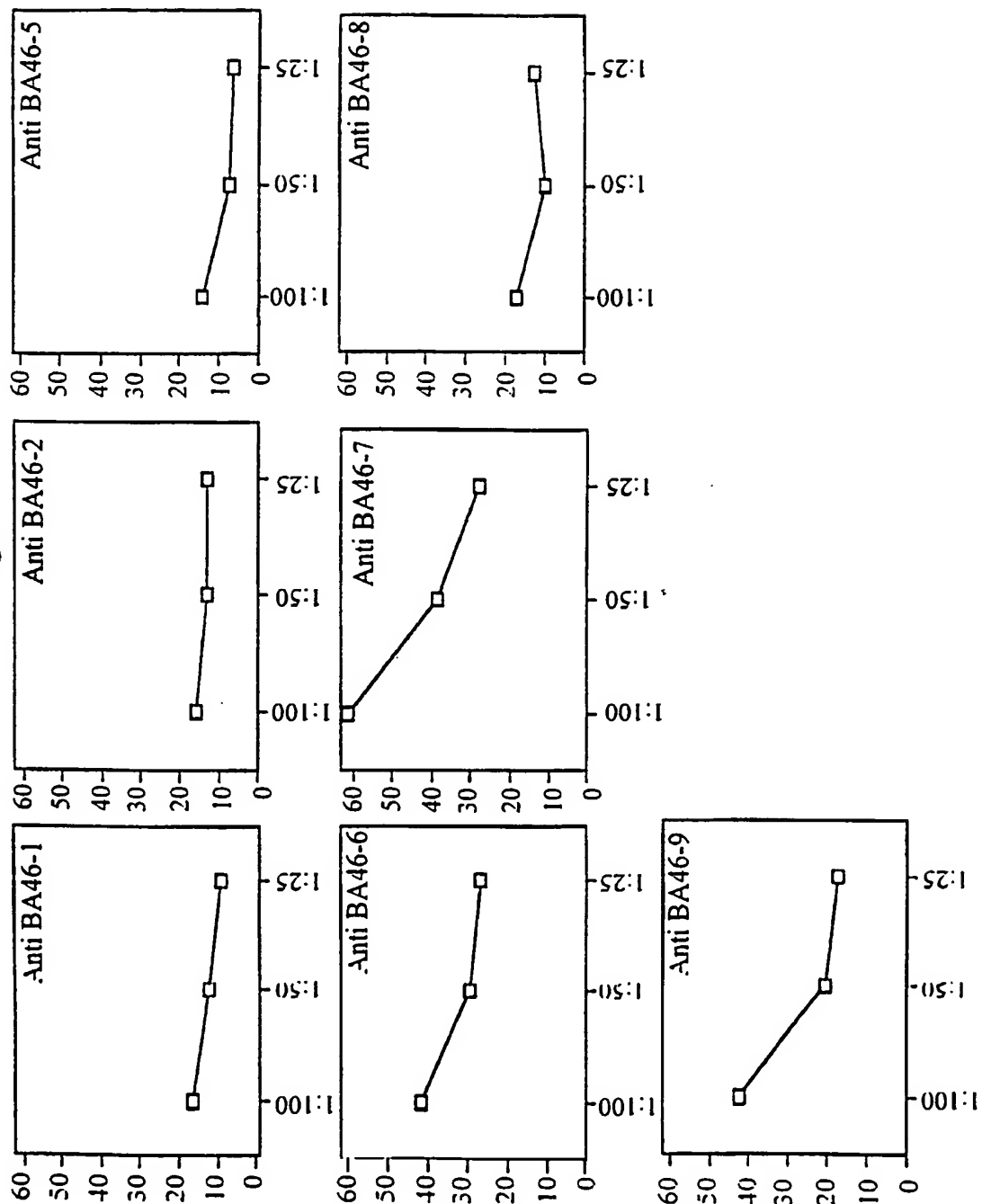


Fig. 13



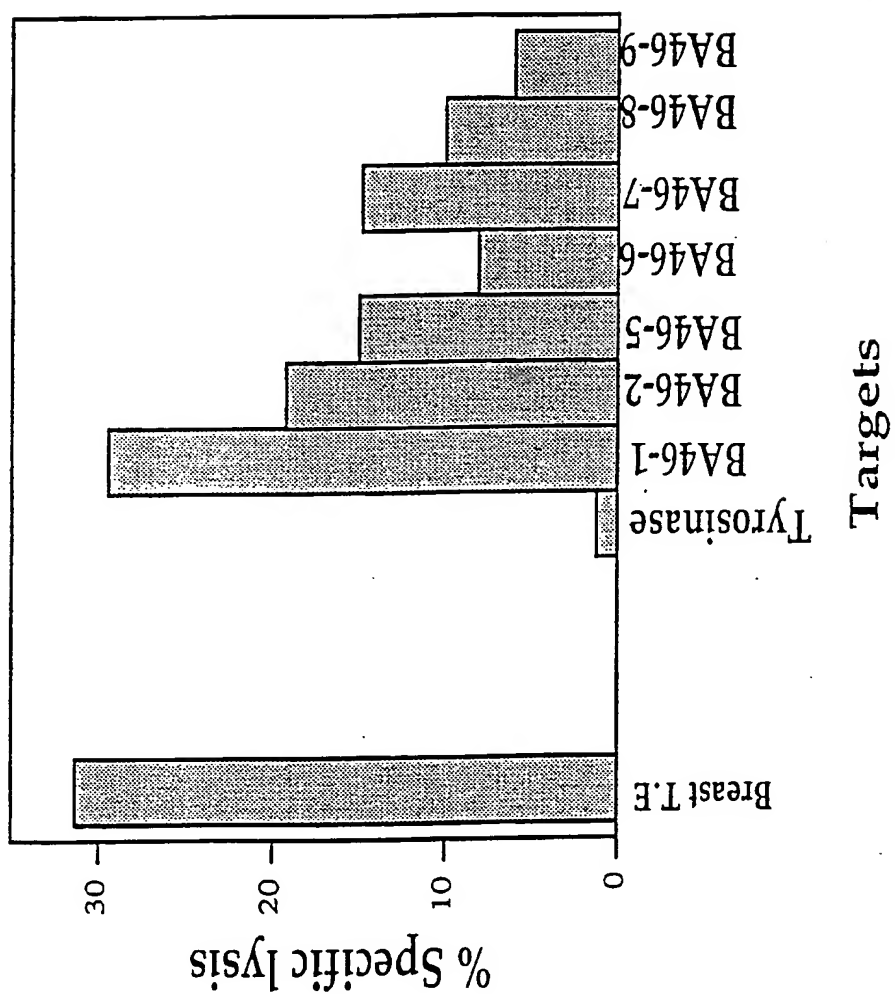
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Fig. 14



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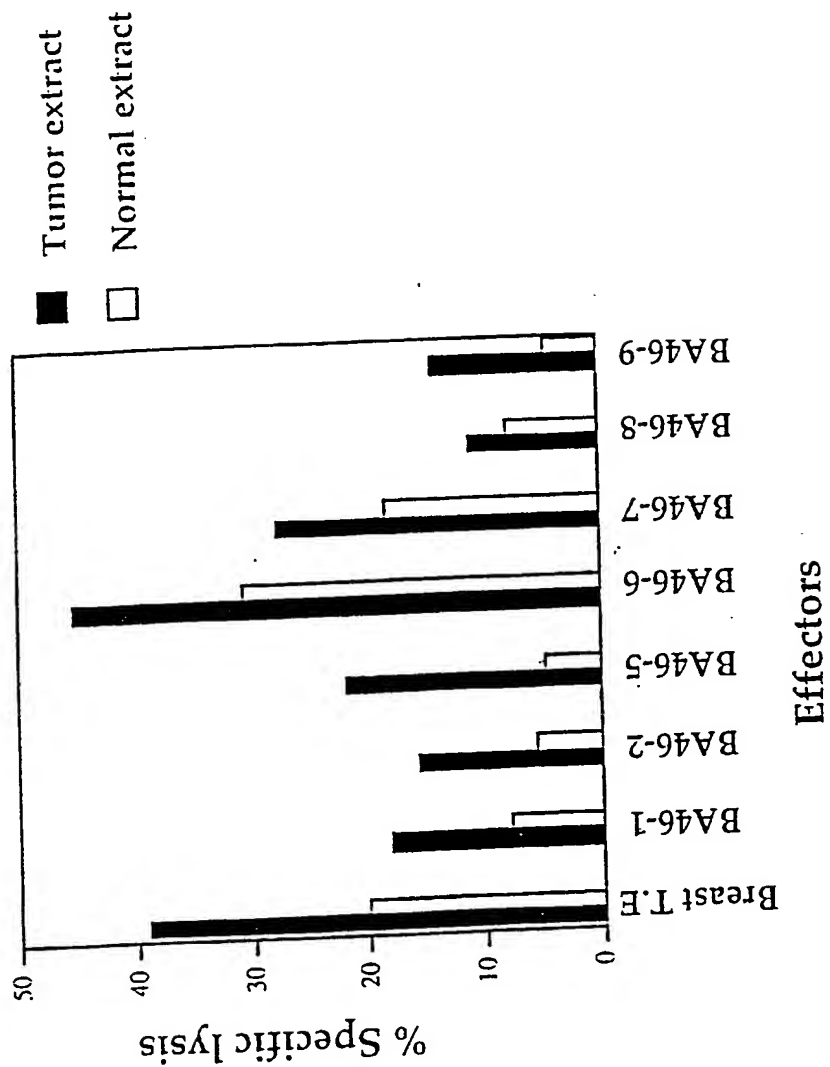
Fig. 15



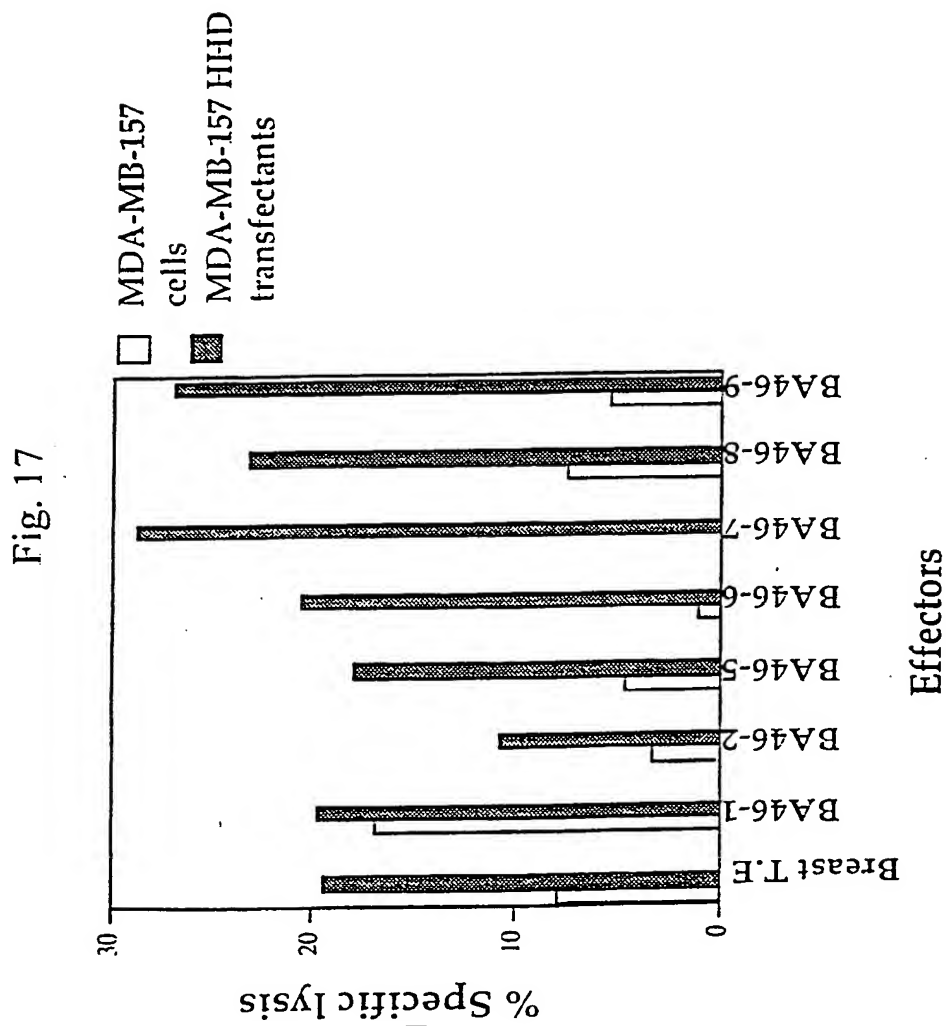


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Fig. 16

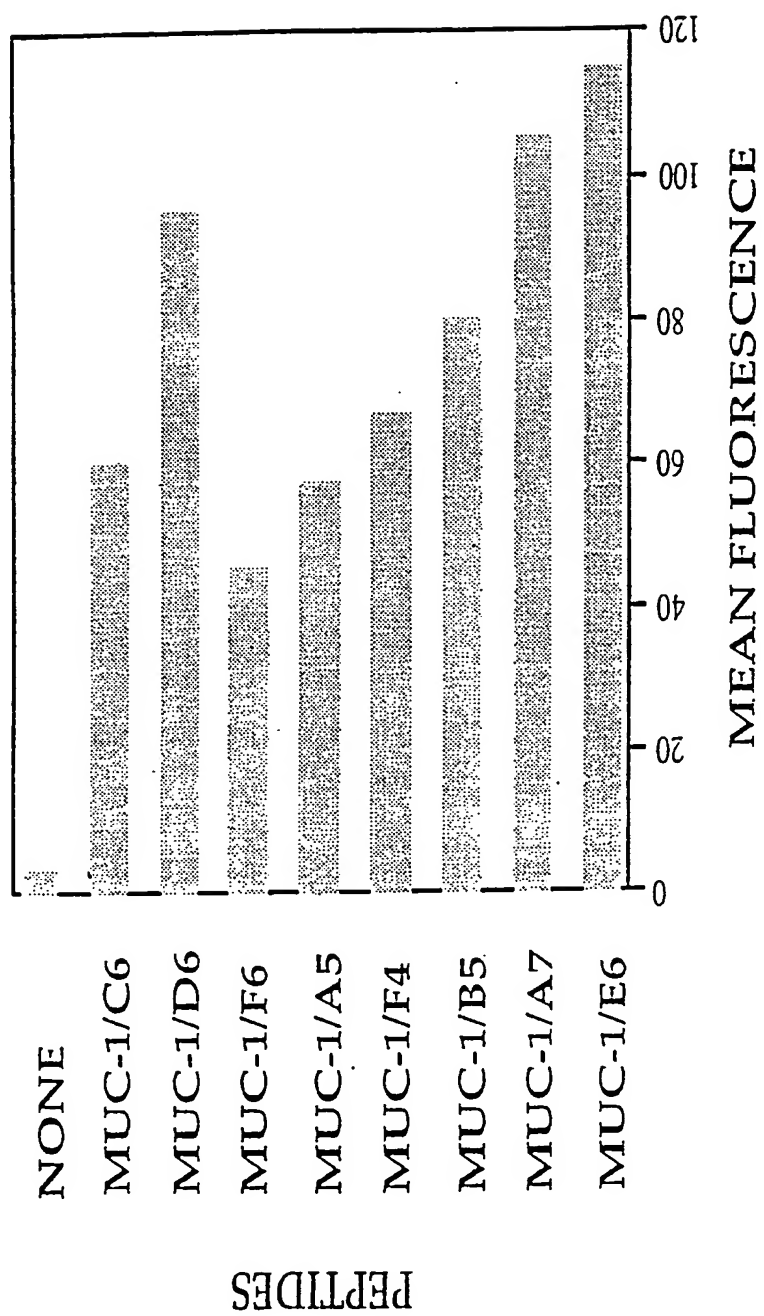


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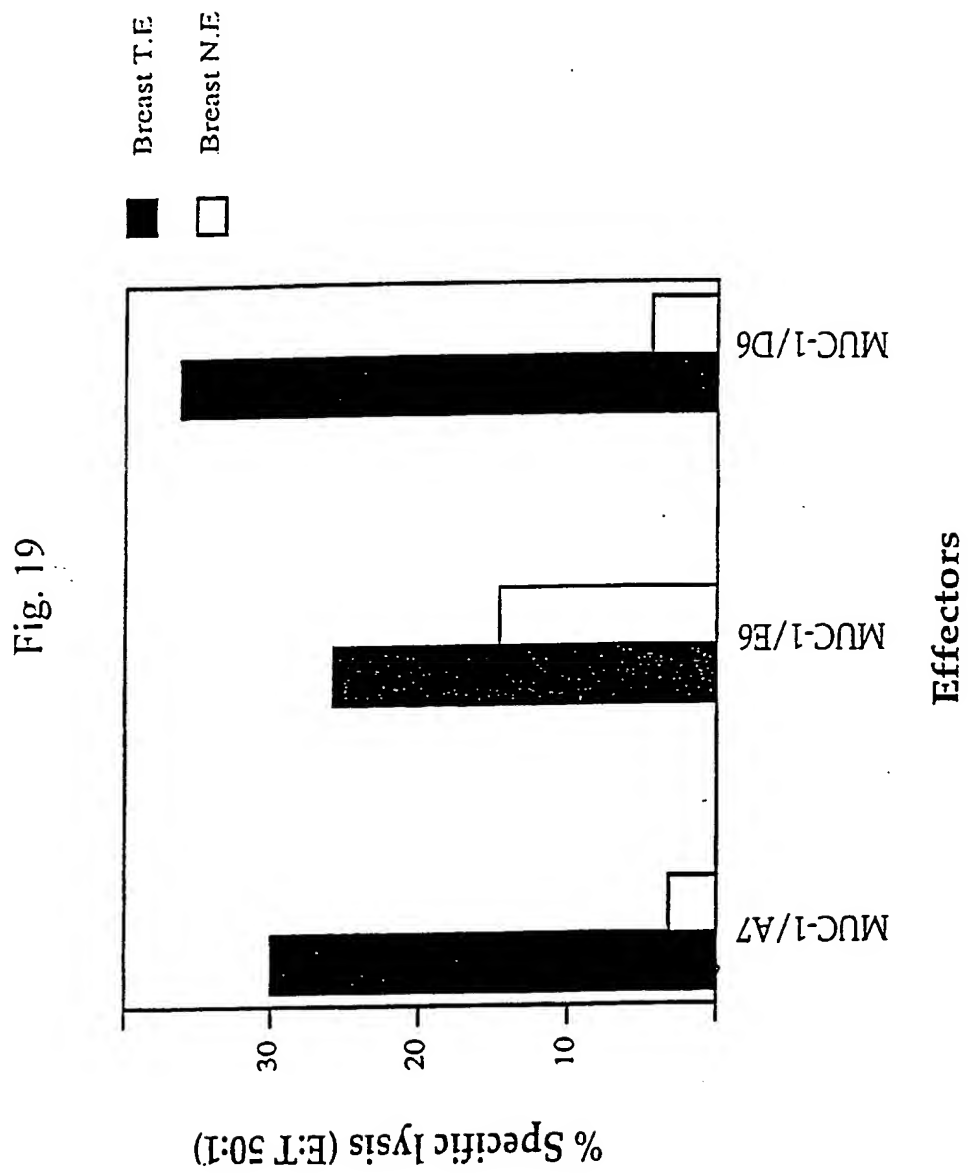


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Fig.18

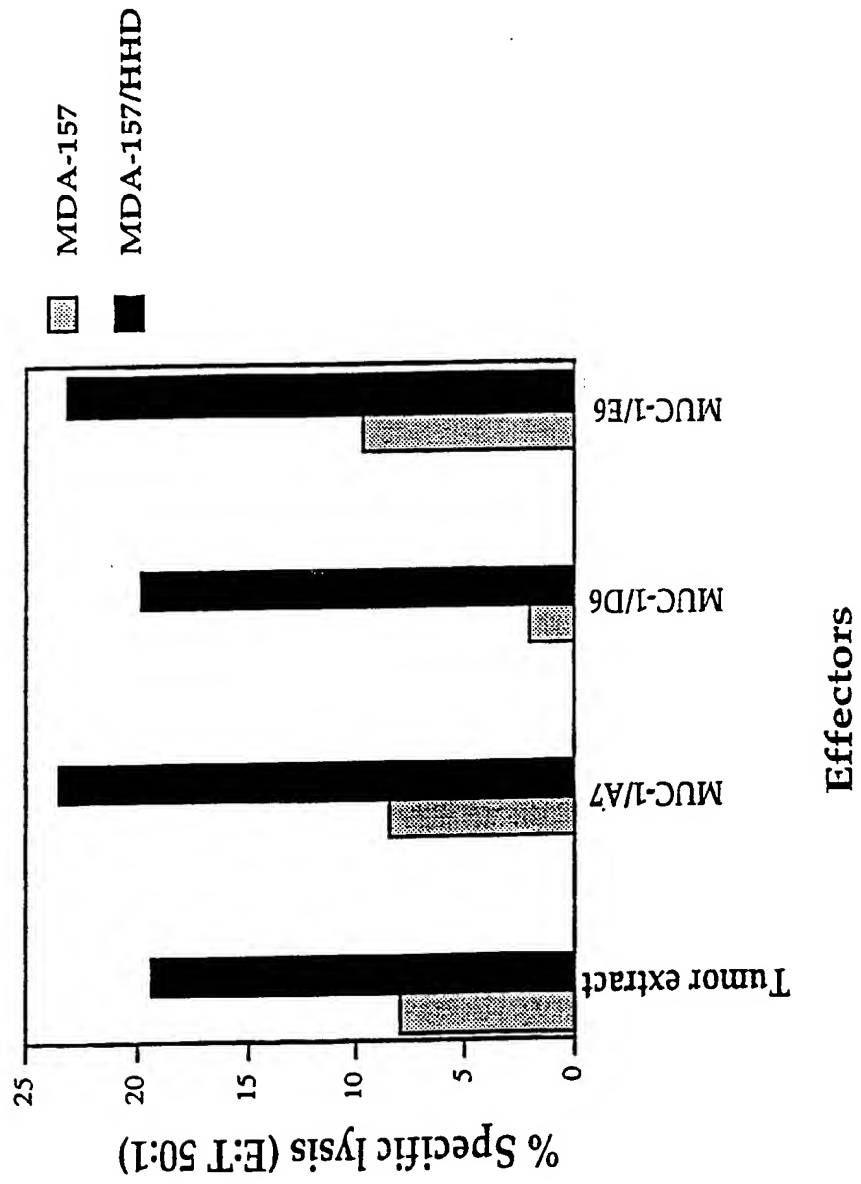


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Fig. 20



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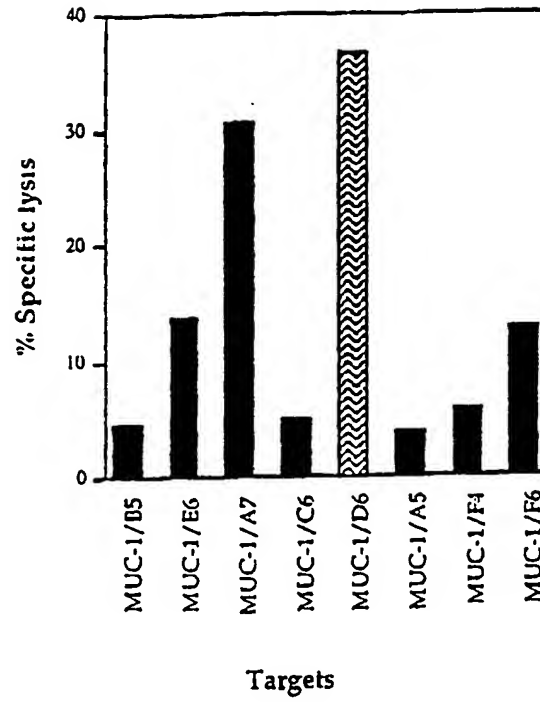


Fig. 21

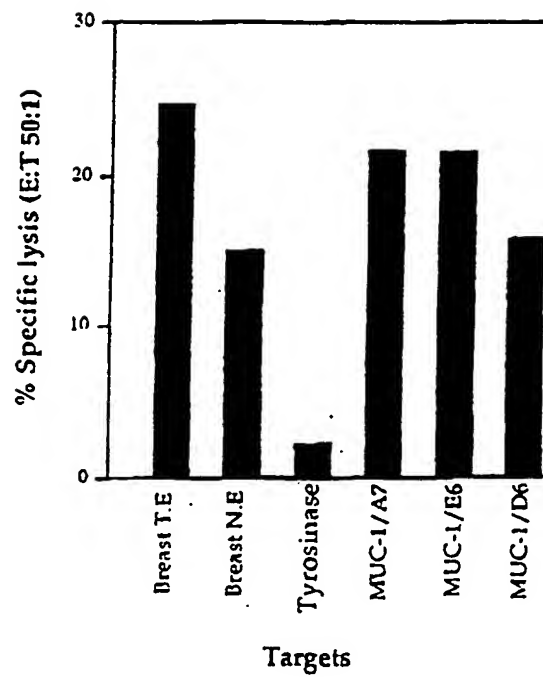


Fig. 22

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Fig. 23a

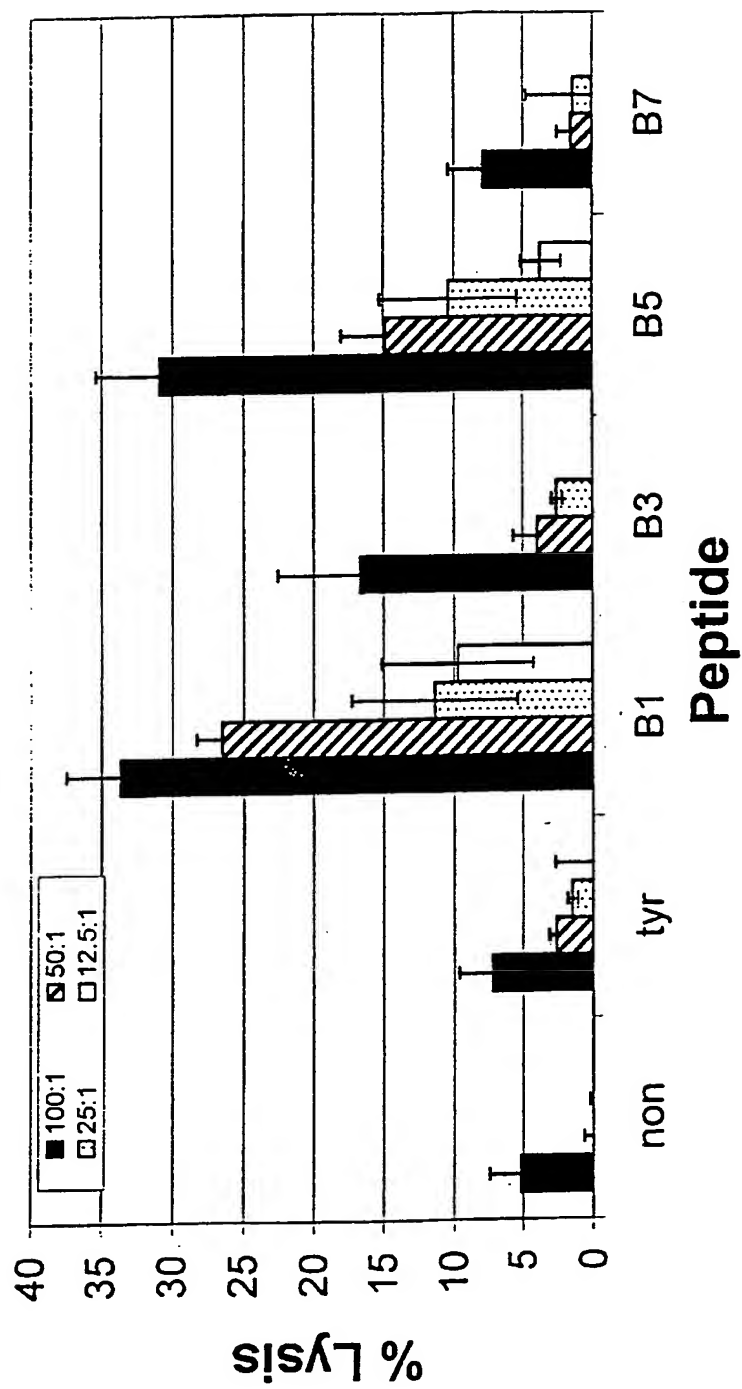
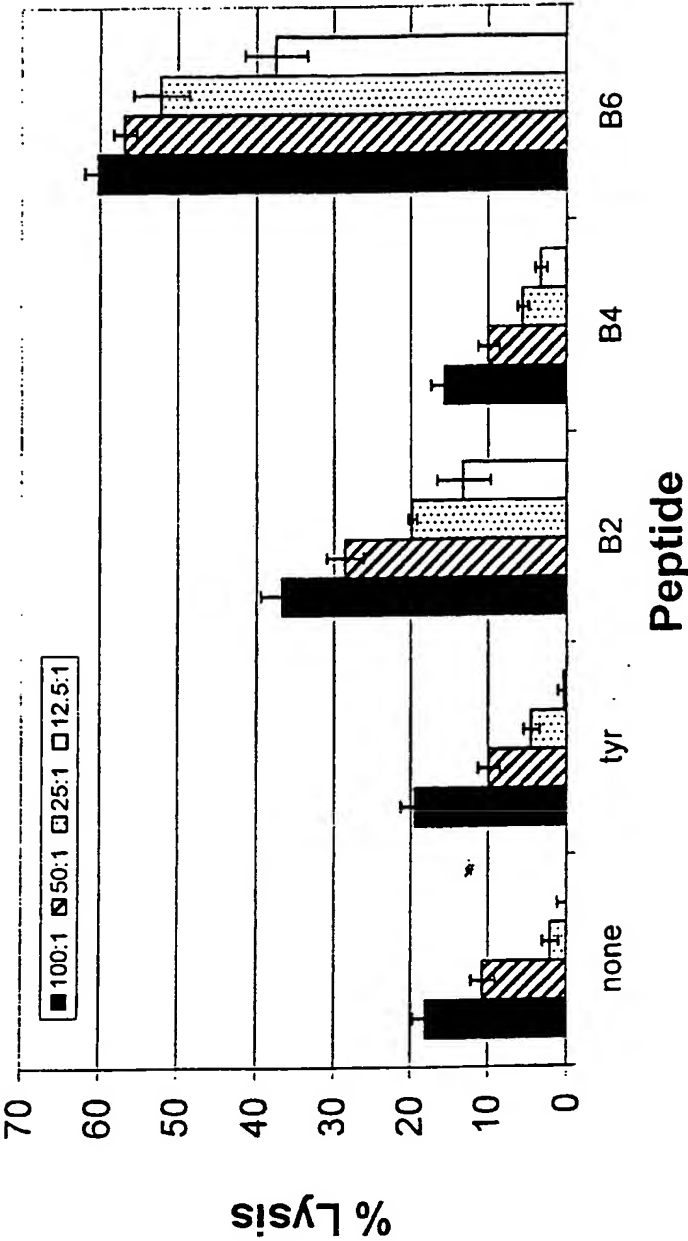


Fig. 23b





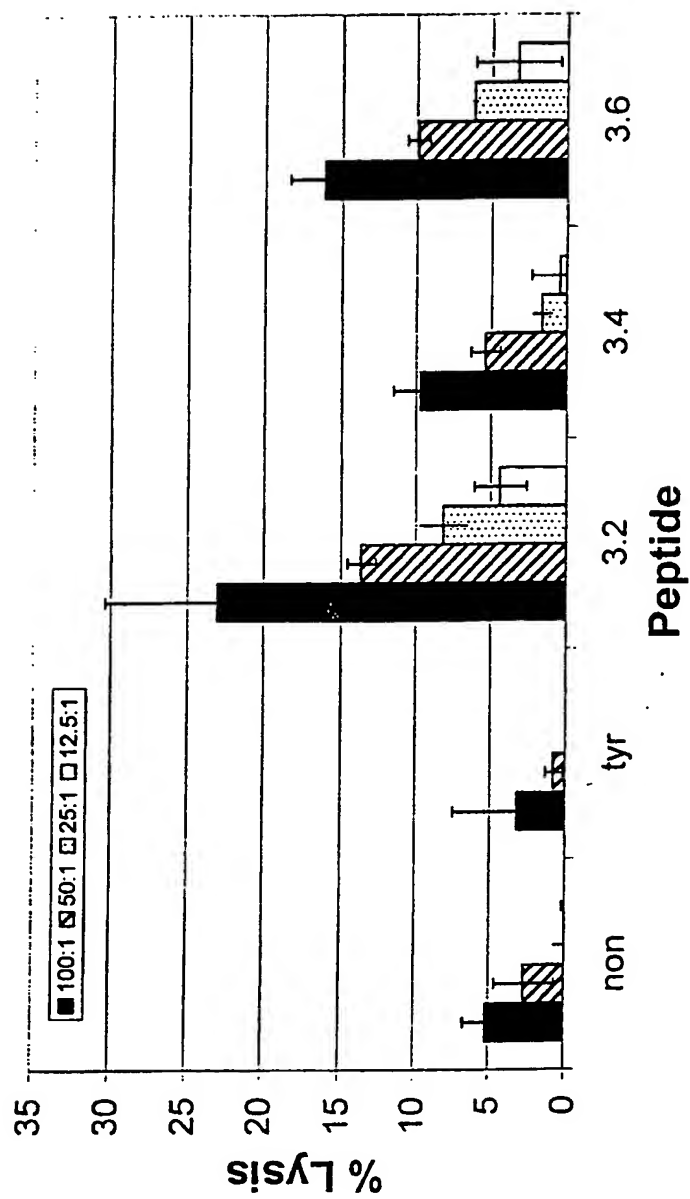
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Fig. 23c



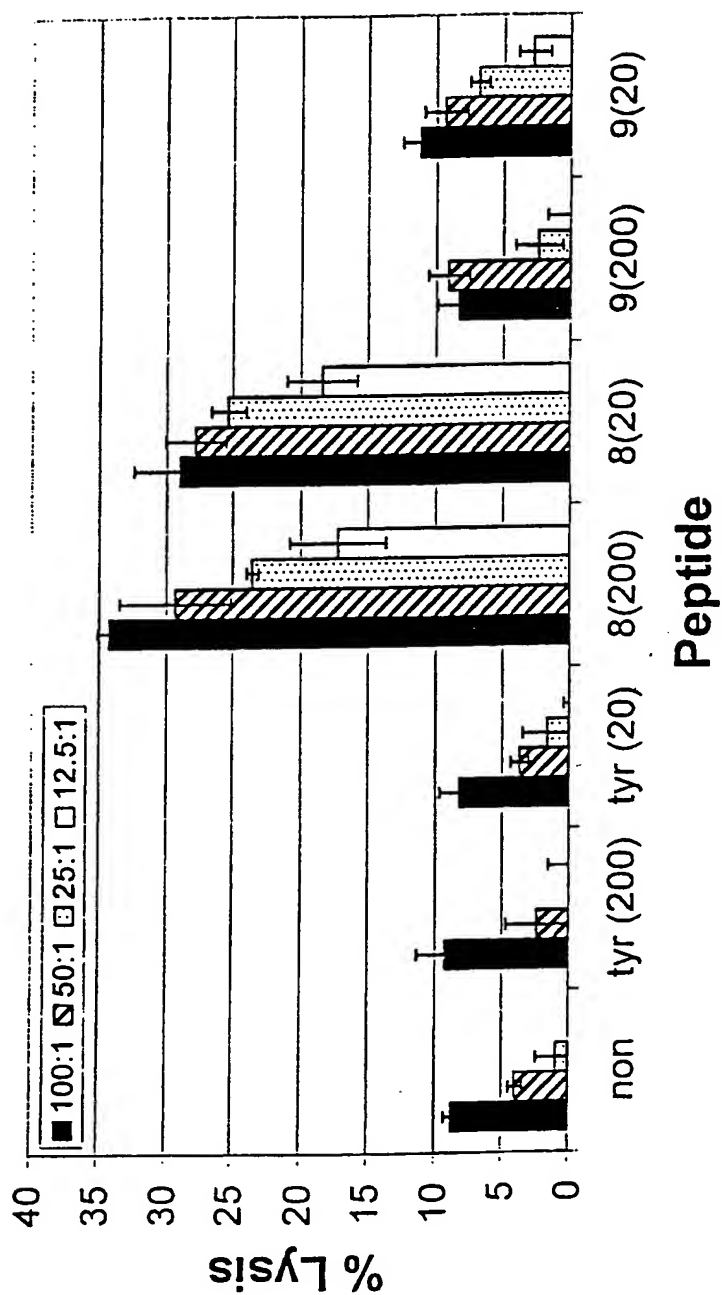
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Fig. 23d



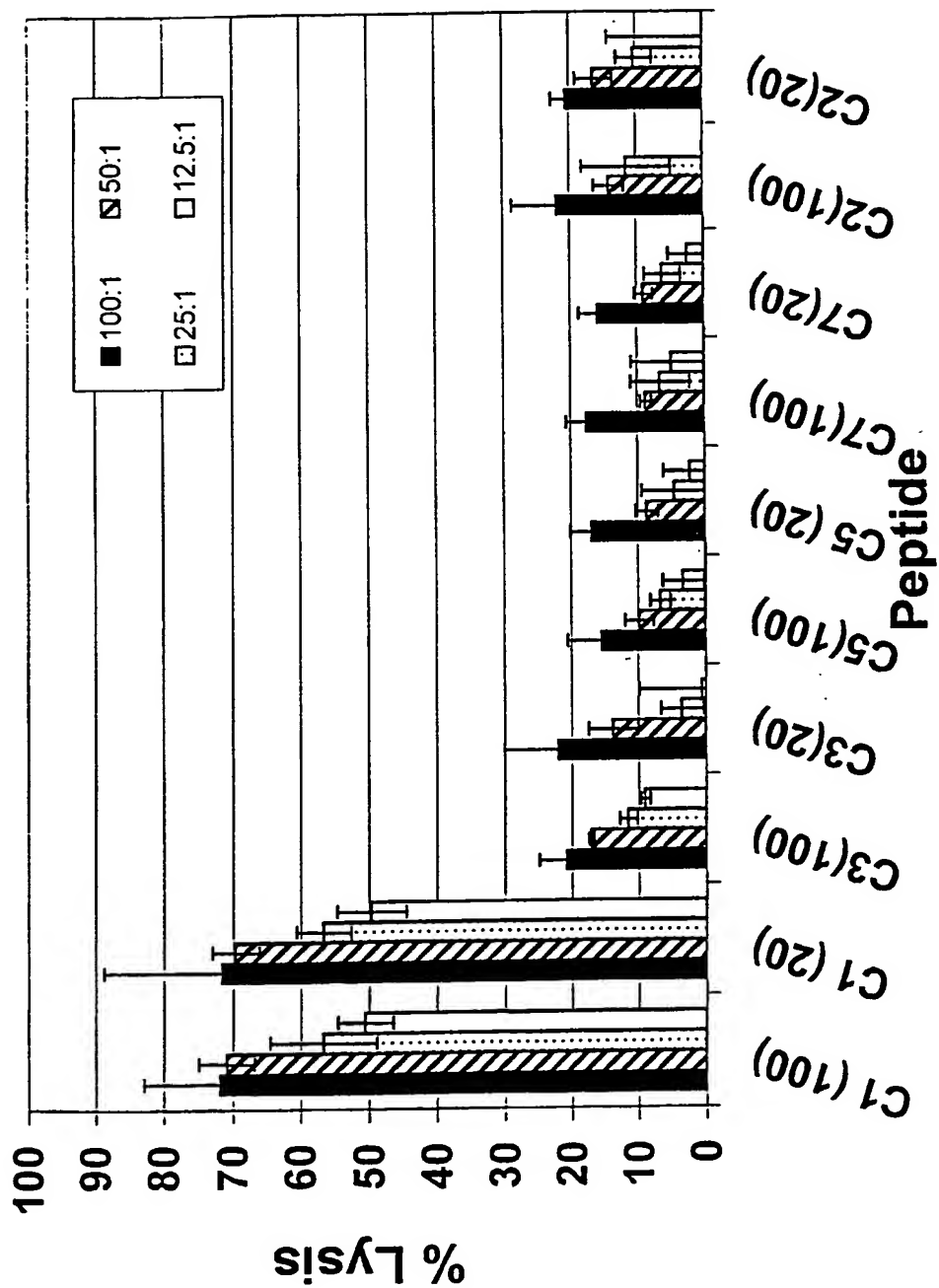
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Fig. 23e



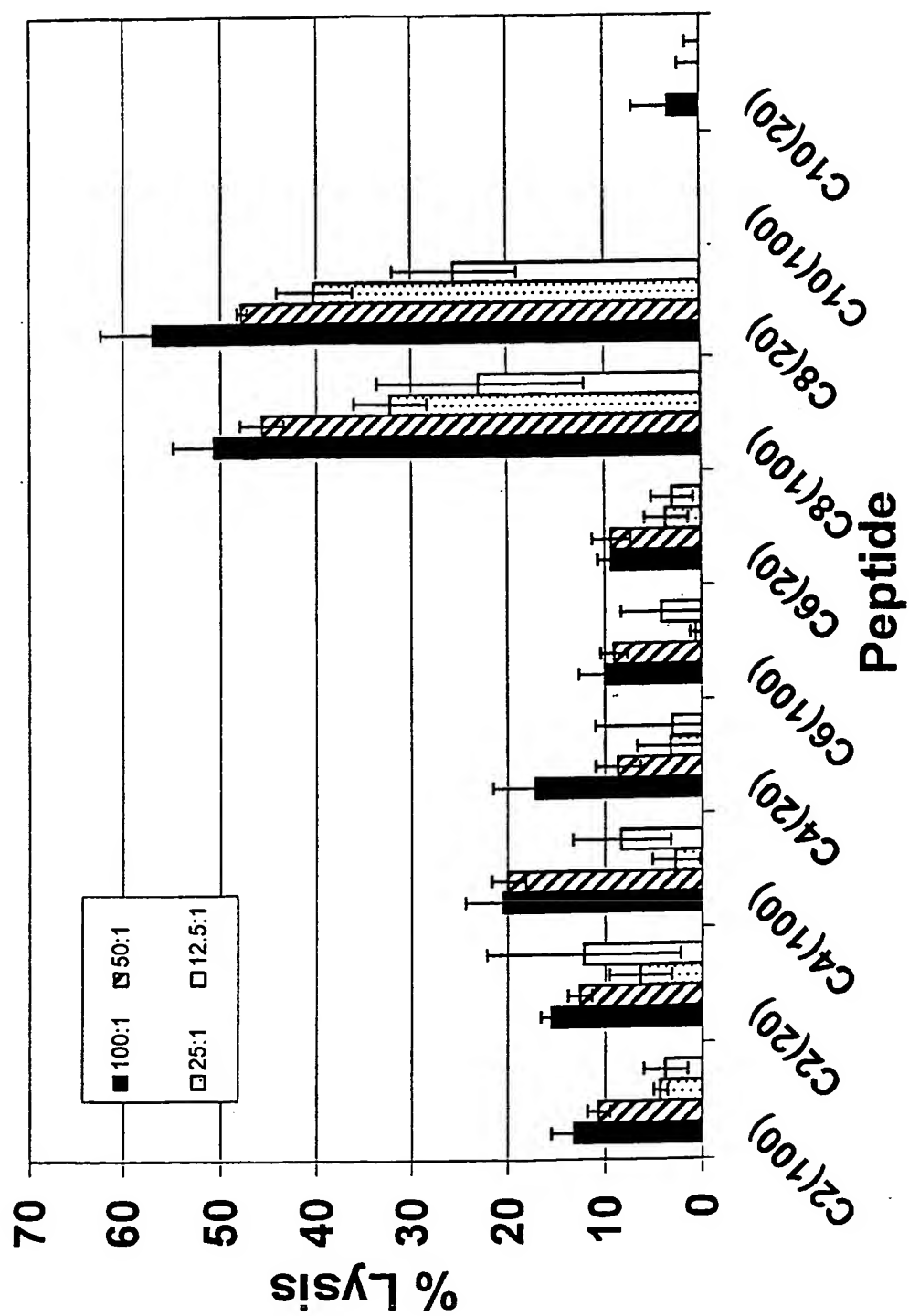
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Fig. 24a



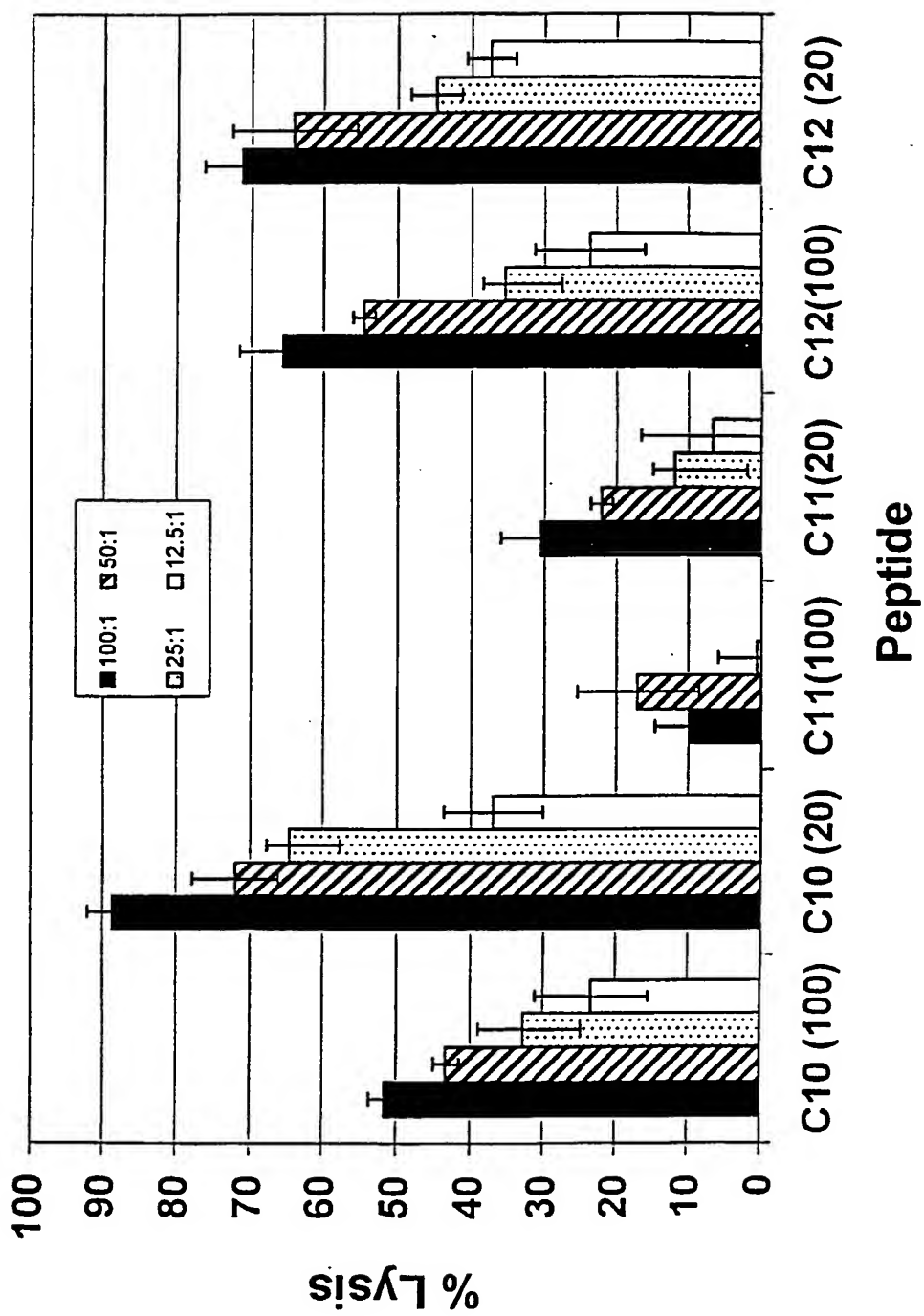
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Fig. 24b



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Fig. 24c



## SEQUENCE LISTING

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/IL 99/00417

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C07K14/47 C07K14/705 C12N9/16 C12N9/64  
A61K38/17 A61K38/46 A61K38/47 C12N15/55 C12N15/57  
C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 98 43084 A (UNIV LELAND STANFORD JUNIOR) 1 October 1998 (1998-10-01) claims; table 4 ---	1, 5, 6, 20-22, 55
X	WO 94 20127 A (CYTEL CORP) 15 September 1994 (1994-09-15) table 25 ---	1, 5, 6, 20-22, 55
X	DE 195 16 673 A (PECHER GABRIELE DR) 31 October 1996 (1996-10-31) claims; examples ---	1, 13, 20-22, 43-45
	--- -/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

30 December 1999

Date of mailing of the international search report

12/01/2000

Name and mailing address of the ISA

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Authorized officer

Fuhr, C

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/IL 99/00417

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 08318 A (CORIXA CORP) 6 March 1997 (1997-03-06)  claims; examples ---	1,5, 20-22, 26,30, 31,34, 51-55
A	WO 95 04548 A (JENNER TECHNOLOGIES) 16 February 1995 (1995-02-16) page 7, line 17 - line 35; claims; examples ---	1,26,32, 37,38,42
P,A	DE 197 58 400 A (HANISCH FRANZ GEORG PROF DR ;MAX DELBRUECK CT FUER MOLEKULA (DE)) 1 July 1999 (1999-07-01) claims; table 1 ---	1,26,32, 37,38,42
X	WO 97 11715 A (AUSTIN RESEARCH INST ;SANDRIN MAURO SERGIO (AU); MCKENZIE IAN FARQ) 3 April 1997 (1997-04-03) claims; examples ---	1,11-13, 26-38, 46,51-55
X	WO 97 35021 A (US HEALTH ;ZAREMBA SAM (US); SCHLOM JEFFREY (US); TSANG KWONG YOK) 25 September 1997 (1997-09-25)  claims; examples; table 10 ---	1,5,20, 21, 26-38, 51-55
A	WO 95 19783 A (CELIS ESTEBAN ;GREY HOWARD M (US); CYTEL CORP (US); KUBO RALPH T ( ) 27 July 1995 (1995-07-27) claims; examples ---	1,26
A	WEINSTEIN B: "CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES AND PROTEINS, PASSAGE" CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES, AND PROTEINS,XX,XX, vol. 7, page 266-357 XP002032461 the whole document -----	23-25

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL 99/00417

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 37-38 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.: 43-45  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/IL 99 00417

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 43-45

Present claims 23-25 relate to an extremely large number of possible compounds/products. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds/products claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds/products prepared in the examples and closely related homologous compounds like those mentioned in the sequence listing.

Present claims 43-45 relate to an extremely large number of possible compounds/products/apparatus/methods. In fact, the claims contain so many options, variables, possible permutations and provisos that a lack of clarity (and/or conciseness) within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, no search has been carried out for this parts of the application.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International publication No

PCT/IL 99/00417

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9843084	A	01-10-1998	US 5858685 A AU 6570998 A	12-01-1999 20-10-1998
WO 9420127	A	15-09-1994	AU 6359494 A AU 6597998 A BR 9406652 A CA 2157510 A CN 1118572 A EP 0703783 A JP 8507525 T NZ 263050 A SG 49008 A	26-09-1994 02-07-1998 10-09-1996 15-09-1994 13-03-1996 03-04-1996 13-08-1996 24-11-1997 18-05-1998
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